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Departamento de Nutrición Vegetal

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TESIS DOCTORAL:

DESARROLLO Y APLICACIÓN DE NUEVAS METODOLOGÍAS ANALÍTICAS AL ESTUDIO DE FERTILIZANTES FÉRRICOS

Memoria presentada por Dña. IRENE ORERA UTRILLA, Licenciada en Químicas, para optar al grado de Doctor en Ciencias.

Dña. Irene Orera Utrilla

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Esta Tesis Doctoral ha sido escrita en la modalidad de compendio de trabajos previamente publicados o aceptados para su publicación y consta de las siguientes publicaciones:

1. Orera Irene, Abadía Javier, Abadía Anunciación, Álvarez-Fernández Ana. Analytical technologies to study the biological and environmental implications of iron-fertilisation using synthetic ferric chelates: the case of Fe(III)-EDDHA – a review. *Journal of Horticultural Science and Biotechnology* (2009) 84(1): 7-12

2. Álvarez-Fernández Ana*, Orera Irene*, Abadía Javier, Abadía Anunciación. Determination of synthetic ferric chelates used as fertilizers by liquid Chromatography-Electrospray/Mass Spectrometry in agricultural matrices. *Journal of the American Society for Mass Spectrometry* (2007) 18: 37-47

3. Orera Irene, Abadía Anuciación, Abadía Javier, Álvarez-Fernández Ana. Determination of *o,o*EDDHA -a xenobiotic chelating agent used in Fe-fertilizers- in plant tissues by liquid chromatography-electrospray mass spectrometry: overcoming matrix effects. *Rapid Communications in Mass Spectrometry* (2009) 23:1694-1702. DOI: 10.1002/rcm.4056.

4. Orera Irene, Orduna Jesús, Abadía Javier, Álvarez-Fernández Ana. Electrospray-collision-induced dissociation mass spectrometry: a tool to characterize synthetic polyaminocarboxylate ferric chelates used as fertilizers. *Rapid Communications in Mass Spectrometry* (2010) 24(1):109-119. DOI: 10.1002/rcm.4361

5. Orera Irene, Rodríguez-Castrillón Jose Ángel, Moldovan Mariella, García-Alonso Jose Ignacio, Abadía Anunciación, Abadía Javier, Álvarez-Fernández Ana. Using a dual-stable isotope tracer method to study the uptake, xylem transport and distribution of Fe and its chelating agent from stereoisomers of a xenobiotic Fe(III)-chelate used as fertilizer in Fe-deficient Strategy I plants. *Metallomics* (enviado).

INFORME DIRECTORES

AUTORIZACIÓN PRESENTACIÓN DE TESIS DOCTORAL EN MODALIDAD DE COMPENDIO DE PUBLICACIONES

Dª. ANA MARÍA ÁLVAREZ FERNÁNDEZ, Científica Titular del Consejo Superior de Investigaciones Científicas (CSIC), y D. JAVIER ABADÍA BAYONA, Profesor de Investigación del mismo organismo.

AUTORIZAN

La presentación en la modalidad de compendio de publicaciones de la siguiente memoria de Tesis Doctoral, titulada "DESARROLLO Y APLICACIÓN DE NUEVAS METODOLOGÍAS ANALÍTICAS AL ESTUDIO DE FERTILIZANTES FÉRRICOS" presentada por Dª IRENE ORERA UTRILLA para optar al grado de Doctor por la Universidad de Zaragoza, y certifican que ha sido realizada bajo nuestra dirección en la Estación Experimental Aula Dei (CSIC).

Y para que conste a los efectos oportunos expedimos la presente autorización

En Zaragoza, a 3 de mayo de 2010

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CERTIFICAN

Que la Tesis Doctoral titulada **"Desarrollo y aplicación de nuevas metodologías analíticas al estudio de fertilizantes férricos"**, ha sido realizada por la Licenciada en Química Dña. IRENE ORERA UTRILLA, en el Departamento de Nutrición Vegetal de la Estación Experimental de Aula Dei del Consejo Superior de Investigaciones Científicas bajo su dirección y reúne, a su juicio, las condiciones requeridas para optar al Grado de Doctor en Ciencias

Zaragoza, mayo de 2010

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Fdo. Javier Abadía Bayona

D. JAVIER GALBÁN BERNAL, Catedrático de Universidad del Departamento de Química Analítica de la Facultad de Ciencias de la Universidad de Zaragoza

CERTIFICA

Que la Tesis Doctoral titulada **"Desarrollo y aplicación de nuevas metodologías analíticas para el estudio de fertilizantes férricos"**, ha sido realizada por la Licenciada en Química Dña. IRENE ORERA UTRILLA, bajo su tutela como ponente en el Departamento de Química Analítica de la Facultad de Ciencias de la Universidad de Zaragoza y, a su juicio, reúne las condiciones requeridas para optar al Grado de Doctor en Ciencias

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ABREVIATURAS

AAS	Espectroscopía de absorción atómica
APCI	Ionización química a presión atmosférica
ATPasa	Adenosina 5’-trifosfato sintasa
BPDS	Ácido 4,7-difenil-1,10-batofenantrolin-disulfónico
CDTA	Ácido ciclohexano-1,2-diaminotetracetico
CID	Disociación inducida por collision
DTPA	Ácido dietilendiaminopentaacético
EDDCHA	Ácido etilendiamino-di-(5-carboxi-2-hidroxifenilacético
EDDHSA	Ácido etiléndiamino-di-(2-hidroxi-5-sulfofenilacético)
EDDS	Ácido etiléndiamino-N,N'-disuccínico
EDTA	Ácido etiléndiaminotetraacético
EI	Impacto electrónico
ESI	Ionización por electrospray
FAB	Bombardeo con átomos acelerados
FCR	Enzima Fe(III)-quelato reductasa
FT-ICR ó FTMS	Ión ciclotrón con transformada de Fourier
FW	Peso fresco
GC	Cromatografía de gases
HEEDTA	Ácido 2-hidroxietiletilendiaminotriacético
HPLC	Cromatografía líquida de alta eficacia
ICP	Plasma de acoplamiento inductivo
IDA	Análisis por dilución isotópica
IDHA	Ácido N-(1,2-dicarboxietil)-D,L-aspártico
IPD	Deconvolución de perfiles isotópicos
IS	Estándar interno
LC	Cromatografía líquida
MALDI	Ionización por desorción láser asistida por matriz
MS	Espectrometría de masas
MS^n	Espectrometría de masas en tandem
m/z	Relación masa/carga
NA	Nicotianamina
NMR	Resonancia magnética nuclear
*o,o*EDDHA	Ácido etiléndiamino-di-(*o*-hidroxifenilacético)
*o,p*EDDHA	Ácido etilendiamino-N-(*o*-hidroxifenilacético)-N’-(*p*-hidroxifenilacético)
*o,o*EDDHMA	Ácido etilendiamino-di-(*o*-hidroxi-*o*-metilfenilacético)
*o,p*EDDHMA	Ácido etilendiamino-di-(o-hidroxi-metilfenilacético)-N’-(p-hidroxi-metilfenilacético)
PS	Fitosideróforo

PI	Patrón interno
Q-TOF	Cuadrupolo-tiempo de vuelo
SPAD	Soil-Plant Analysis Development
TOF	Tiempo de vuelo

ÍNDICE

La estructura de esta Tesis Doctoral se ajusta a la normativa aprobada el 17 de diciembre del 2008 por la Universidad de Zaragoza y que regula la estructura de las tesis como compendio de publicaciones.

Capítulo 1

INTRODUCCIÓN

ÍNDICE

1. LA DEFICIENCIA DE HIERRO EN PLANTAS

El hierro (Fe) es un micronutriente esencial para las plantas, animales y otros organismos, ya que es un constituyente indispensable de un gran número de enzimas que intervienen en algunas de las principales funciones del metabolismo de los seres vivos. En las plantas, el Fe participa en la síntesis de clorofila y es esencial para la fotosíntesis y el mantenimiento de la estructura del cloroplasto (Abadía *et al.*, 2004). Además, interviene en procesos como la respiración, la fijación de nitrógeno (Clark, 1983) y en la síntesis de ADN y hormonas (Briat y Lobreaux, 1997).

La deficiencia de hierro es una alteración nutricional de las plantas muy extendida que se conoce habitualmente con el nombre de clorosis férrica. El término clorosis, en sentido general, significa falta de clorofila en un órgano vegetal, lo cual produce una pérdida o disminución de su color verde (Figura 1-1). La clorosis producida por deficiencia de Fe (clorosis férrica) se manifiesta por un amarilleamiento que aparece en las zonas intervenales de las hojas jóvenes, mientras que los nervios permanecen verdes. La clorosis puede ser causada también por la insuficiencia en el suministro de otros elementos esenciales para la planta (Mn, Mg, Zn, etc). Alternativamente, puede ser debida, directa o indirectamente, a situaciones de estrés como el déficit hídrico o el ataque de patógenos.

Figura 1-1. Síntomas de la deficiencia de Fe en melocotonero (*Prunus persica*, izquierda) y tomate (*Lycopersicum esculentum*, derecha)

Esta alteración nutricional afecta a una alta variedad de especies y regiones geográficas como España, Francia, Italia, Turquía, EE.UU., etc. Las especies más susceptibles a sufrir clorosis férrica son la soja (*Glycine max*), cacahuete (*Arachis hypogaea*), frijol (*Phaseolus vulgaris*), sorgo (*Sorghum bicolor*), arroz (*Oryza sativa*), cítricos, melocotonero, vid (*Vitis vinifera*) y tomate. La principal consecuencia de la deficiencia de Fe en plantas es la disminución en la producción agrícola que se debe sobre todo a la reducción en la producción de fotosintatos necesarios para el crecimiento y desarrollo de la planta (Miller *et al.*, 1984). En el caso de árboles frutales, el efecto sobre la producción es especialmente significativo, ya que disminuye tanto el número como la calidad del fruto (Álvarez-Fernández *et al.*, 2003; Álvarez-Fernández *et al.*, 2006). Esto se debe a un acortamiento del ciclo vital del árbol, ya que su etapa productiva resulta considerablemente inferior a la normal. Otra consecuencia importante de esta deficiencia es que las plantas son la principal fuente de Fe en la dieta de una gran parte de la población mundial, por lo que los bajos niveles de Fe en plantas afectan también a la salud humana. De hecho, según la

Organización Mundial de la Salud (OMS) 2 billones de personas en el mundo tienen anemia (Santi y Schmidt, 2009).

Por último, el impacto económico que supone la clorosis férrica en la agricultura es muy importante, debido no solo al descenso en la producción y valor nutricional de las partes comestibles de la planta, sino también al elevado precio de algunos fertilizantes férricos (estimado en 200-400 € ha^{-1} para árboles frutales (Rombolà y Tagliavini, 2006).

1.1 Causas de la deficiencia de Fe

La deficiencia de Fe es un problema complejo, cuyas causas se deben generalmente a la combinación de varios factores:

Baja disponibilidad de Fe en los suelos

Aunque los suelos normalmente contienen cantidades altas de Fe, la concentración de Fe disponible en solución es muy baja. Esto ocurre principalmente en suelos alcalinos y calizos (pH 7,5-8,5) en los que el Fe se encuentra en forma de óxidos e hidróxidos de baja solubilidad (Lindsay y Schwab, 1982). Se ha estimado que, aproximadamente, el 30% de los suelos cultivados en el mundo son calcáreos (Mori, 1999). Su elevado contenido en caliza ($CaCO_3$) produce un efecto tampón que dificulta la solubilización de los óxidos e hidróxidos de Fe. Por lo tanto, los factores que determinan la disponibilidad de Fe en el suelo son el pH, el tipo de mineral del que procede el Fe, ya que cuanto más soluble sea el mineral, mayor concentración de Fe habrá disponible en la solución del suelo, y la superficie específica del mineral, ya que cuanto menor sea ésta más lenta será la liberación del Fe (Schwertmann, 1991).

La presencia del ión bicarbonato en los suelos influye negativamente en la biodisponibilidad del Fe ya que puede afectar al crecimiento y metabolismo de las raíces (Yang *et al.*, 1994), al transporte del Fe a la parte aérea y a la disponibilidad fisiológica del Fe en las hojas (Alhendawi *et al.*, 1997). Además, la posible alcalinización del apoplasto y citoplasma, debida a la presencia de bicarbonato, puede causar la precipitación del Fe en dichos compartimentos (Romera *et al.*, 1992) y una disminución de la actividad reductasa férrica de la raíz (Susín *et al.*, 1996).

Asimismo, otras características del suelo como la elevada proporción de arcilla, falta de materia orgánica, alta humedad, poca aireación, elevada salinidad y alta concentración de fosfatos, afectan negativamente a la disponibilidad del Fe (Lindsay y Schwab, 1982; Shenker y Chen, 2005).

Factores relacionados con la absorción y metabolismo del Fe

Dentro de estos factores destacan los que i) inhiben el crecimiento de las raíces tales como la compactación y encharcamiento del suelo (Chen y Barak, 1982), las bajas temperaturas y la adición de herbicidas (Chaney, 1984), y los que ii) inhiben la absorción del Fe como la presencia de fosfatos y metales pesados (Lucena, 2003).

Factores que afectan a la actividad del Fe dentro de la planta

Se ha observado en frutales crecidos en el campo que el contenido de Fe en las hojas de plantas deficientes puede ser similar o incluso algo superior al de las hojas verdes y, sin embargo, presentar síntomas de deficiencia de Fe. Este fenómeno es conocido como la "paradoja de la clorosis férrica" (Morales *et al.*, 1998) y sugiere que el Fe podría acumularse en alguna zona de la hoja (principalmente en los nervios principal y

secundarios (Jiménez *et al.*, 2009)) en una forma no utilizable por la planta. Además, se ha propuesto que los fosfatos y un elevado pH del apoplasto podrían provocar la precipitación del Fe en el exterior de la célula impidiendo su utilización (Mengel y Geurtzen, 1986).

1.2 Adquisición de hierro por la raíz en condiciones de deficiencia de hierro

Las plantas toman el Fe directamente de la disolución del suelo mediante un proceso de absorción activa a través de las raíces (Uren, 1984). El Fe es absorbido preferentemente en forma de Fe(II) por todas las plantas excepto las gramíneas, que lo toman como Fe(III) quelado. Cuando la cantidad de Fe a disposición de la planta es suficiente, las plantas tienden a utilizar sistemas de transporte de Fe de baja afinidad y a absorber sólo la cantidad necesaria para un óptimo crecimiento, previniendo así una posible toxicidad. El Fe libre puede ser altamente tóxico, ya que reacciona con oxígeno y forma radicales libres que dañan componentes celulares como ADN, proteínas, lípidos y azúcares (Hell y Stephan, 2003). Cuando la cantidad de Fe disponible para la planta no es suficiente, las plantas pueden permanecer indiferentes (plantas *no eficientes*) o desarrollar mecanismos de adaptación para aumentar su capacidad de adquirir Fe del suelo (plantas *eficientes*). Dependiendo del mecanismo de adaptación desarrollado para adquirir Fe en condiciones de deficiencia, las plantas se dividen en dos grupos distintos: plantas de Estrategia I y plantas de Estrategia II. Dentro de cada grupo el grado de respuesta es diferente entre especies e incluso entre genotipos.

1.2.1 Estrategia I

Las plantas dicotiledóneas y monocotiledóneas no gramíneas pertenecen a la Estrategia I y se caracterizan por desarrollar en condiciones de deficiencia de Fe una serie de respuestas que afectan a la fisiología, estructura y morfología de las raíces, todas ellas encaminadas a aumentar la capacidad de absorción del Fe del suelo. En cuanto a los cambios fisiológicos destacan (Figura 1-2):

1. La reducción del Fe(III) a Fe(II) por medio de la enzima Fe(III)-quelato reductasa localizada en la membrana plasmática de las células de la raíz (FCR o FRO2 (Robinson *et al.*, 1999)).
2. Transporte del Fe(II) al interior de la célula a través del transportador IRT1 (Eide *et al.*, 1996; Vert *et al.*, 2002).
3. Excreción de H^+ a la rizosfera por medio de una H^+-ATPasa con objeto de acidificar la rizosfera y mejorar la solubilidad del Fe(III) (Santi y Schmidt, 2009).

La enzima Fe(III)-quelato reductasa (FRO2) es una proteína transmembrana que pertenece a la familia del flavocitocromo *b*. Su topología ha sido descrita en Arabidopsis (*Arabidopsis thaliana*; (Schagerlof *et al.*, 2006)) y consiste en 8 hélices transmembrana y un “loop” citosólico soluble que contiene motivos de unión a NADPH, FAD y secuencias con homologías con oxidorreductasas. En el caso de Arabidopsis, se han descrito hasta siete genes distintos pertenecientes a la familia FRO que codifican polipéptidos con sitios de unión para FAD y NAD(P)H, los cuales actúan como donadores de electrones para reducir el Fe^{3+} externo (Robinson *et al.*, 1999). Estos genes han sido también identificados en otras especies de Estrategia I incluyendo guisante (*Pisum sativum*), tomate y pepino (*Cucumis sativus*; Jeong y Connolly, 2009). Curiosamente, mientras la expresión del *FRO2* en

Arabidopsis está restringida a las capas externas de la raíz, consistente con su papel de toma de Fe del suelo, la expresión del gen *FRO1* en guisante se ha visto a lo largo de toda la raíz (incluyendo los nódulos) y hojas; sugiriendo que los FROs pueden estar además involucrados en la distribución del Fe en la planta (Jeong y Connolly, 2009).

Una vez reducido, el Fe(II) es transportado al interior de las células epidérmicas de la raíz mediante el transportador de metales divalentes IRT1 (Morrissey y Guerinot, 2009). Aunque el IRT1 puede transportar otros metales como Zn, Mn, Co, Cd y Ni presenta una alta afinidad por el Fe(II) y se expresa bajo deficiencia de Fe. Además, si existe un aporte suficiente de Fe, esta proteína se degrada evitando la toxicidad (Connolly *et al*., 2002).

La acidificación de la rizosfera, al contrario de lo que ocurre con la reducción férrica, no es evidente en todas las especies de Estrategia I y, en aquellas especies que la muestran, esta respuesta depende de muchos factores como el balance de incorporación cationes/aniones, la composición de los exudados de la raíz y el tipo de nutrición nitrogenada. La acidificación de la rizosfera se realiza por medio de la H^+-ATPasa que tiene como objeto, además de solubilizar el Fe(III), prevenir la repulsión de los quelatos de Fe cargados negativamente de la pared celular e intervenir en la regulación de la expresión de los genes *FRO2* e *IRT1* (Santi y Schmidt, 2009). En Arabidopsis, se conocen 12 isoformas diferentes de la H^+-ATPasa, de las cuáles la AHA2 es la principal responsable de la acidificación de la rizosfera y la AHA7 aparece asociada al desarrollo de pelos radiculares, que es un cambio morfológico inducido en respuesta a la deficiencia de Fe (Santi y Schmidt, 2009).

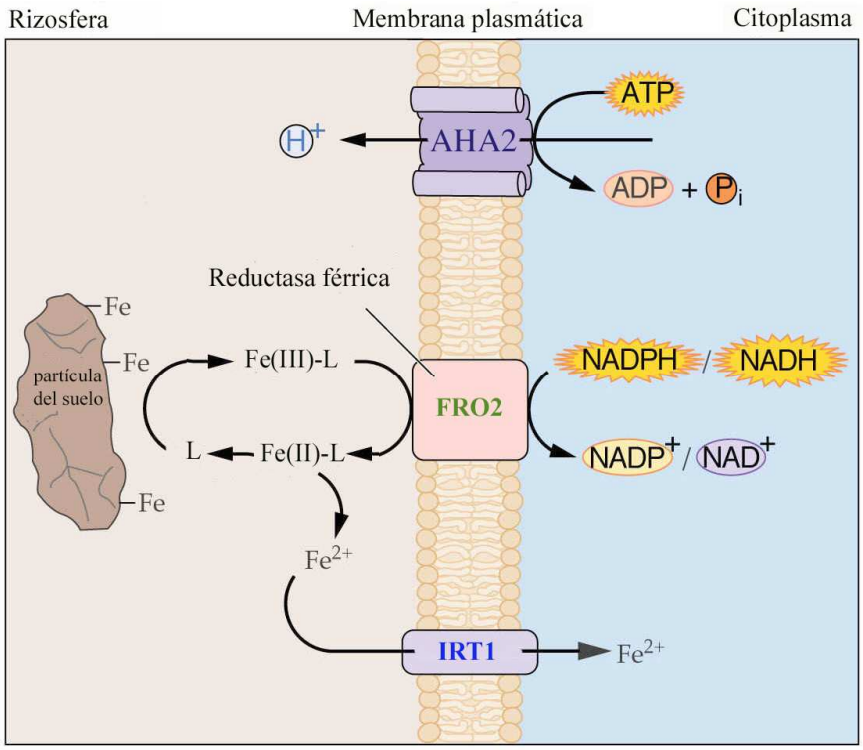

Figura 1-2. Estrategia I (Modificada de (Buchanan *et al*., 2000)). L representa un ligando orgánico.

Otro cambio fisiológico que presentan las plantas en condiciones de deficiencia de Fe es la excreción de compuestos de bajo peso molecular, con el fin de aumentar la

biodisponibilidad del Fe del suelo por complejación (formación de compuestos organo-férricos solubles), o bien por reducción de las formas insolubles de Fe en el suelo (oxi hidróxidos férricos). Entre los compuestos de bajo peso molecular excretados se encuentran los ácidos orgánicos, principalmente citrato y malato, los fenoles como el ácido cafeico, ácido *p*-cumárico, ácido fenólico y el ácido clorogénico, los flavonoides y las flavinas (Robin *et al.*, 2008). La función de los ácidos orgánicos consiste en formar complejos estables con Fe(III) favoreciendo así su solubilización en el suelo (Schmidt, 1999). A los fenoles se les atribuyó inicialmente una función reductora del Fe(III), sin embargo recientemente se ha demostrado que tienen un papel importante en la removilización del Fe apoplástico de la raíz (Jin *et al.*, 2007). Los exudados de flavonoides como la genisteína, quercetina y campferol están relacionados con la reducción de Fe(III) y en algunos casos, como la miricetina y la quercetina, forman complejos estables con Fe(III) (Cesco *et al.*, 2010). En condiciones de deficiencia de Fe, algunas especies acumulan y excretan flavinas, principalmente la riboflavina y, en algunas plantas como remolacha (*Beta vulgaris* L.) y espinaca (*Spinacia oleracea* L.), los sulfatos de riboflavina (Susín *et al.*, 1993; Susín *et al.*, 1994). El papel de las flavinas en la deficiencia de Fe es todavía desconocido aunque existen hipótesis como que estos compuestos sean parte integral de los sistemas de reductasa férrica de las raíces o tengan una acción antimicrobiana en las proximidades de la raíz, disminuyendo la posibilidad de que los microorganismos del entorno compitan con la planta por la adquisición de Fe (Susín *et al*., 1993).

Entre los cambios morfológicos de la raíz en condiciones de deficiencia de Fe destacan: una disminución en el crecimiento de la raíz principal (Römheld y Marschner, 1981b), un aumento en el número de raíces laterales y pelos radiculares (Moog *et al*., 1995) y un engrosamiento en las zonas subapicales de la raíz (López-Millán et al., 2000). Estos cambios morfológicos van encaminados al aumento de la superficie de contacto entre la raíz y el suelo, incrementando de esta forma la posibilidad de adquisición de Fe por la planta.

1.2.2 Estrategia II

Las plantas gramíneas producen y secretan a la rizosfera compuestos de bajo peso molecular llamados fitosideróforos (PS). Debido a su alta afinidad por el Fe, los PS quelan y solubilizan de forma muy eficaz el Fe(III) del suelo, formando complejos Fe(III)-fitosideróforo (Fe(III)-PS) que son tomados por la raíz a través de un sistema de transporte de alta afinidad (Figura 1-3).

Los PS son derivados del ácido mugénico (Takagi, 1976). En la Figura 1-4 se muestran las estructuras de dos fitosideróforos. Su mecanismo de excreción todavía no está claro, pero se ha sugerido que se produce a través de vesículas o canales aniónicos (Negishi *et al.*, 2002). Una vez excretados a la rizosfera, los PS quelan el Fe(III) del suelo, formando el complejo Fe(III)-PS. La entrada del complejo Fe(III)-PS al citoplasma de la célula se realiza a través de una proteína específica llamada YS1 localizada en las zonas apicales de la raíz (Curie *et al.*, 2001). Esta proteína está formada por 682 aminoácidos con 12 dominios transmembrana, y pertenece a una nueva subclase de la familia de transportadores oligopeptídicos OPT (Curie *et al.*, 2001). YS1 está regulada por la deficiencia de Fe en la raíz y el tallo y permite también el transporte del complejo Fe(II)-nicotianamina (Fe(II)-NA), un importante transportador de Fe(II) en la planta (Hell y Stephan, 2003). Una vez que el complejo se encuentra en el citosol, el Fe(III) es liberado y el PS se degrada o se excreta nuevamente al exterior. Se ha comprobado que, bajo deficiencia de Fe, la velocidad de absorción del PS libre y el Fe(III)-PS aumenta, pero muestran diferente velocidades de

absorción, lo que sugiere que las células de la raíz podrían diferenciar entre el PS libre y el Fe(III)-PS (Kawai y Alam, 2006).

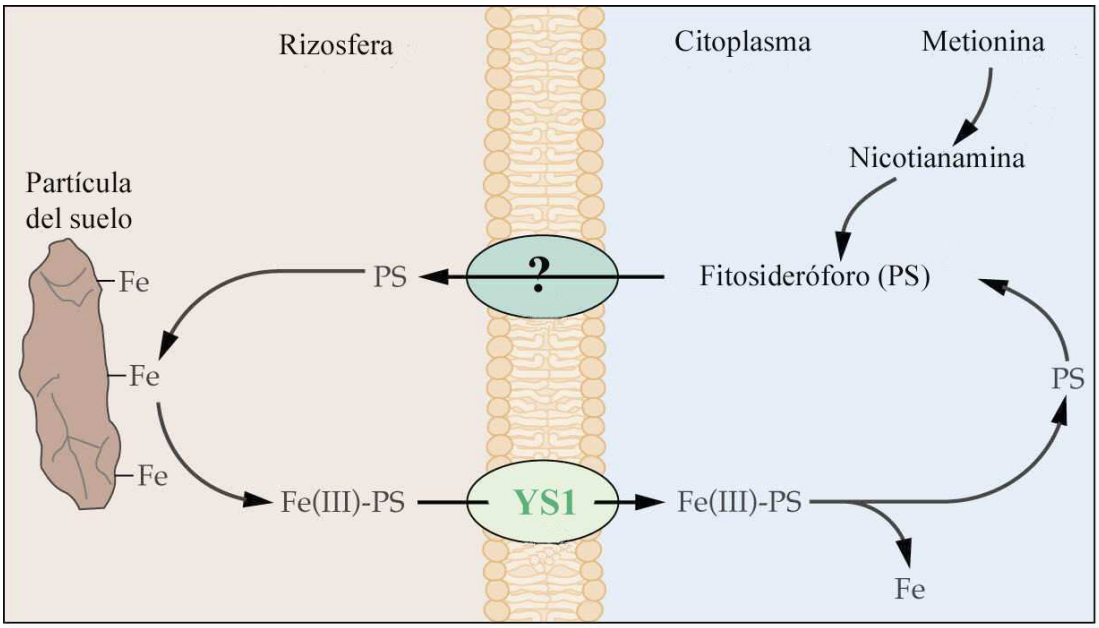

Figura 1-3. Estrategia II (Modificada de Buchanan *et al*., 2000)

Las plantas de Estrategia II son menos sensibles al pH del suelo que las de Estrategia I, existiendo una alta correlación entre la cantidad de PS excretados y la resistencia a la deficiencia de Fe (Morrissey y Guerinot, 2009). Sin embargo, algunas plantas gramíneas como el arroz combinan componentes de ambas estrategias. De hecho, la cantidad de PS que excreta el arroz en condiciones de deficiencia de Fe es menor que la de otras plantas gramíneas como el maíz (*Zea mays* L.) o la cebada (*Hordeum vulgare* L.), pero se ha observado una mayor expresión de los transportadores de metales divalentes OsIRT1 y OSIRT2 (Morrissey y Guerinot, 2009).

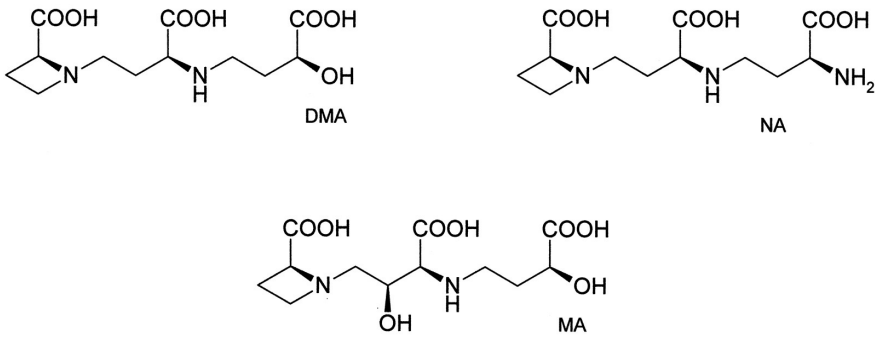

Figura 1-4. Estructuras de algunos fitosideróforos. DMA: ácido 2-desoxi mugineico. NA: nicotianamina. MA: Ácido mugineico

1.3 Corrección de la deficiencia de Fe

Existen diferentes técnicas de prevención y tratamiento de la clorosis férrica.

Antes de establecer el cultivo la mejora y selección genética de cultivares resistentes a la clorosis férrica es una herramienta muy útil que permite solventar esta deficiencia mediante el cultivo de cultivares resistentes, obtenidos tanto por técnicas tradicionales de selección como mediante modificación genética (Charlson *et al.*, 2003). Sin embargo, es frecuente que las variedades tolerantes sean altamente sensibles a otros tipos de estrés (bióticos y/o abióticos) o presenten menor productividad y calidad de los frutos (Tagliavini y Rombolà, 2001). Por ello, la selección de cultivares suele responder más a criterios de productividad y aceptación en el mercado que a su susceptibilidad a sufrir clorosis férrica (Rombolà y Tagliavini, 2006).

En cultivos ya establecidos, el empleo de prácticas agrícolas apropiadas resulta crucial para prevenir la aparición de la clorosis férrica. Estas prácticas consisten en eliminar los riesgos de compactación, alcalinización y encharcamiento del suelo favoreciendo un buen drenaje (Chen y Barak, 1982). Otra práctica habitual en árboles frutales y viñedos, es su cultivo junto con otras plantas anuales, reduciendo así la compactación y aumentando la porosidad, filtración y contenido de materia orgánica del suelo (Tagliavini y Rombolà, 2001). Entre estas plantas anuales se incluye el cultivo de especies con Estrategia II que excretan fitosideróforos capaces de aumentar la solubilidad del Fe en el suelo (Rombolà y Tagliavini, 2006). La adición de enmendadores como la materia orgánica (sustancias húmicas) y acidificantes se lleva a cabo para favorecer la solubilización del Fe nativo no disponible del suelo.

Actualmente, la adición de productos fertilizantes es la técnica más eficaz y común para corregir deficiencias de Fe en cultivos ya establecidos. Estos se pueden clasificar en compuestos de Fe inorgánicos ($FeSO_4$, $Fe_2(SO_4)_3$, piritas, etc.) y compuestos de Fe orgánicos. Dentro de los compuestos de Fe orgánicos podemos distinguir entre los de origen natural y los sintéticos.

Los agentes complejantes de origen natural permitidos para su uso en aplicaciones foliares y en fertirrigación en España incluyen: el ácido lignosulfónico, ácido glucónico, ácido heptaglucónico, sustancias húmicas, aminoácidos libres y el ácido cítrico (Rodríguez-Lucena, 2010). Los lignosulfonatos son polímeros naturales que se obtienen como subproducto de la industria del papel y pueden formar complejos con metales. Los complejos comerciales de metales con lignosulfonatos se han aplicado frecuentemente en el campo con resultados variables. Para el caso de la clorosis férrica han demostrado ser eficaces en peral cuando los complejos eran aplicados por vía foliar (Raese y Staiff, 1988). Las sustancias húmicas se originan a partir de la transformación en el suelo de residuos animales y vegetales. Son sustancias ricas en grupos fenólicos y carboxílicos que pueden formar complejos con el Fe. Estas moléculas han demostrado ser eficaces recuperando plantas de pepino (Estrategia I) y cebada (Estrategia II) deficientes en Fe (Cesco *et al.*, 2002).

Los agentes quelantes sintéticos son los más utilizados, a pesar de su elevado coste, para corregir la deficiencia de Fe en cultivos establecidos, sobretodo en cultivos de alto valor económico. La razón principal es que presentan una eficacia extraordinaria a la hora de suministrar Fe a la planta gracias a su elevada capacidad de mantener el Fe en solución incluso a pH alcalinos. Los quelatos férricos más comunes son el Fe(III)-EDTA, Fe(III)-*o,o*EDDHA, Fe(III)-DTPA, Fe(III)-EDDHSA y Fe(III)-*o,o*EDDHMA. Sin embargo la

creciente preocupación ambiental de los últimos años debido a su escasa biodegradabilidad ha llevado a proponer y evaluar nuevos quelatos sintéticos más biodegradables como correctores de la clorosis férrica. Entre ellos cabe destacar el ácido iminodisuccínico (IDHA) y el ácido etilendiaminodisuccínico (EDDS; Rodríguez-Lucena *et al*. 2010 y Villén *et al.* 2007).

2. QUELATOS FÉRRICOS SINTÉTICOS EN AGRICULTURA

Los quelatos férricos se usan en el campo para prevenir y corregir la clorosis férrica de cultivos sensibles y en fertirrigación o hidroponía para mantener el Fe soluble en la disolución.

2.1 Características de los quelatos férricos sintéticos

Los quelatos metálicos (incluyendo los férricos) están formados por aniones orgánicos complejos, llamados agentes quelantes o ligandos, con dos o más grupos funcionales capaces de compartir pares de electrones con un ión metálico central (Shenker y Chen, 2005). De esta manera, se forma una estructura cíclica en la que el metal (en este caso el Fe) queda retenido, mejorando así su solubilidad y estabilidad y por consiguiente también su biodisponiblidad.

Los agentes quelantes permitidos por la legislación europea (EU Regulations Nº 162/2007; Anon, 2007) para su uso como fertilizantes de Fe y otros micronutrientes son derivados de los siguientes ácidos poliaminocarboxílicos:

Ácido etilendiaminotetraacético	EDTA
Ácido 2-hidroxietiletilendiaminotriacético	HEEDTA
Ácido dietilendiaminopentaacético	DTPA
Ácido etilendiamino-di-(*o*-hidroxifenilacético)	*o,o*EDDHA
Ácido etilendiamino-N-(*o*-hidroxifenilacético)-N'-(*p*-hidroxifenilacético)	*o,p*EDDHA
Ácido etilendiamino-di-(*o*-hidroxi-metilfenilacético)	*o,o*EDDHMA
Ácido etilendiamino-di-(*o*-hidroxi-metilfenilacético)-N'-(*p*-hidroxi-metilfenilacético)	*o,p*EDDHMA
Ácido etilendiamino-di-(5-carboxi-2-hidroxifenilacético)	EDDCHA
Ácido etilendiamino-di-(2-hidroxi-5-sulfofenilacético) y sus productos de condensación	EDDHSA

y se pueden clasificar dependiendo de la presencia o no de grupos fenólicos en su estructura (Figura 1-5). Por una parte se encuentran los agentes quelantes que tienen una estructura similar a la del EDTA, que se denominan *no fenólicos*. Por otro lado están los que se parecen estructuralmente al *o,o*EDDHA, que se denominan *fenólicos*.

Estos agentes quelantes presentan al menos seis grupos funcionales donadores de electrones en su estructura; en el caso de los quelatos *no fenólicos*, al menos cuatro grupos carboxílicos y dos aminos, y en el caso de los quelatos *fenólicos* dos grupos carboxílicos, dos aminos y dos fenólicos. Estos grupos donadores de electrones hacen que el Fe(III) se

coordine octaédricamente con los agentes quelantes hexadentados, obteniéndose un compuesto con estructura cíclica que protege al Fe(III) de su precipitación y del ataque de agentes oxidantes. Algunos agentes quelatantes tienen isómeros posicionales dependiendo de si los grupos –OH se encuentran en posición *orto-orto (o,o)* u *orto-para (o,p)* (Figura 1-5). En el caso de estos últimos, el Fe está unido a través de sólo 5 enlaces al ligando y la sexta posición la ocupa una molécula de H_2O o un OH dependiendo del pH, ya que el grupo hidroxílo fenólico situado en posición *para* está en una posición que no le permie la coordinarse con el Fe.

El EDDHA y sus compuestos homólogos (EDDHMA, EDDHSA y EDDCHA) poseen, además, isomería óptica ya que tienen dos carbonos quirales que pueden ser (RR), (SS), (RS) o (SR). Los isómeros (RS) y (SR) son imágenes especulares superponibles, por lo que se consideran la misma molécula, que se denomina isómero *meso*. Los isómeros (RR) y (SS) son imágenes especulares no superponibles que difieren únicamente en la dirección hacia la que desvían el plano de luz polarizada (enantiómeros). Debido a la difícil separación de estas dos moléculas por métodos químicos, generalmente, se les denomina mezcla *d,l-racémica* (de aquí en adelante la llamaremos isómero *racémico*), si bien se ha visto en los cristales de esta mezcla que el isómero SS contribuye menos del 1% a la totalidad de la mezcla *d,l-racémica* (Bailey *et al.*, 1981).

Las diferencias entre los diastereoisómeros de estos quelatos férricos (*racémico* y *meso*) han sido estudiadas en el Fe(III)-*o,o*EDDHA y el Fe(III)-*o,o*EDDHMA, basándose fundamentalmente en su comportamiento cromatográfico, cristalográfico y su espectro UV-visible. Ya en 1962, Hill-Cottingham separó mediante cromatografía de papel el Fe(III)-EDDHA en dos bandas, una roja y otra violeta, las cuales se identificaron como los isómeros *meso* y *racémico*, aunque no pudieron asignar cada banda a un isómero concreto (Hill-Cottingham, 1962). La separación de ambos isómeros y la asignación de cada pico a su correspondiente isómero se realizó por cromatografía de intercambio aniónico (Barak y Chen, 1987) y cromatografía de par iónico (Lucena *et al.*, 1996). La separación de los isómeros geométricos del Fe(III)-EDDHMA se consiguió también por cromatografía de par iónico (Deacon *et al.*, 1994; Hernández-Apaolaza *et al.*, 1997). Estudios de cristalografía mostraron que la sal magnésica de Fe-EDDHA contenía exclusivamente el isómero *racémico* (Bailey *et al.*, 1981), característica que fue utilizada posteriormente para separar de diastereoisómeros por precipitación (Yunta *et al.*, 2003b). Los espectros de UV-vis de estos quelatos *fenólicos* presentan tres máximos de absorción a aproximadamente 210, 280 y 480 nm, que corresponden al anillo de benceno, al grupo hidroxilo en *orto* de dicho anillo (Jaffe y Orchin, 1962) y al enlace Fe-fenolato, respectivamente (Álvarez-Fernández *et al.*, 2002).

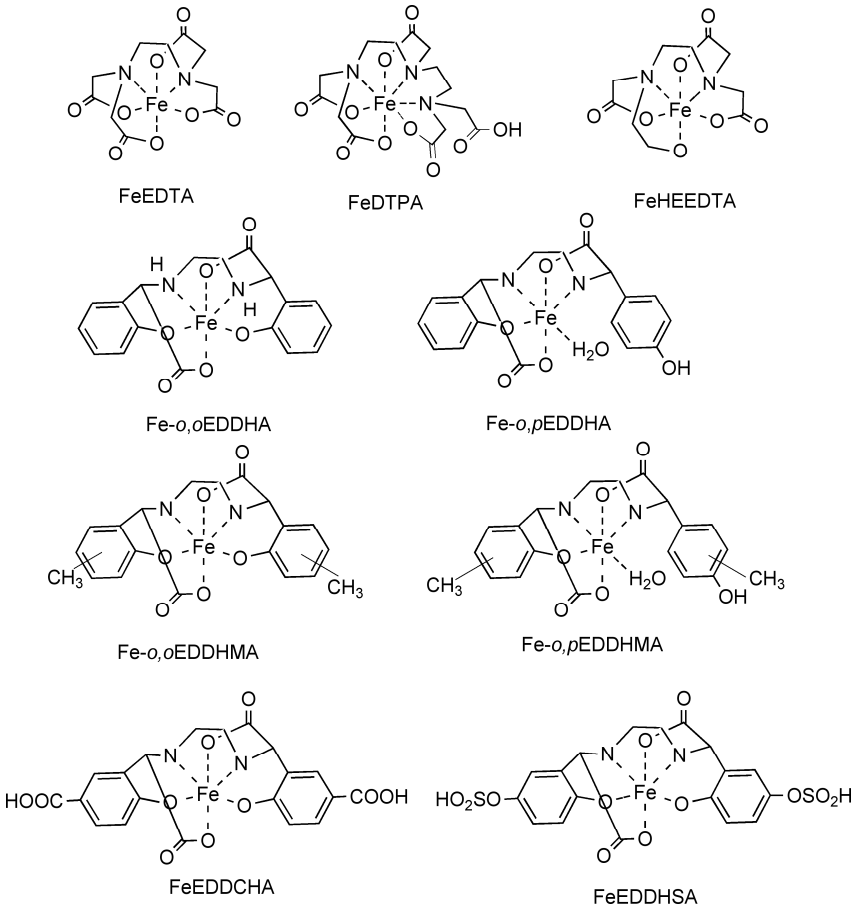

Figura 1-5. Estructuras químicas de los quelatos férricos autorizados según la Regulación Europea EU Nº 162/2007 (Anon, 2007). Por simplicidad de la Figura no se han representado los productos de condensación del EDDHSA.

La constante de formación de un quelato determina la afinidad de la molécula de agente quelante por el metal. Las constantes de estabilidad de los diferentes agentes quelantes con Fe se muestran en la Tabla 1-1.

Tabla 1-1. Logaritmo de las constantes de estabilidad ([FeL]/[Fe]·[L]) del Fe(III) con EDTA, DTPA y HEEDTA (Martell y Smith, 1974) y con EDDHA, EDDHMA y EDDHSA (Yunta *et al.*, 2003b).

Agente quelante	Log K	Agente quelante	Log K	Agente quelante	Log K
EDTA	25,0	*o,o*EDDHA	35,1	*o,o*EDDHMA	34,4
DTPA	27,3	*racemic-o,o*EDDHA	35,9	*racemic-o,o*EDDHA	33,7
HEEDTA	19,8	*meso-o,o*EDDHA	34,1	*meso-o,o*EDDHA	35,5
		*o,p*EDDHA	28,7	EDDHSA	32,8

Como se puede observar en la tabla, las constantes de estabilidad de los quelatos férricos *fenólicos* son mayores que las de los *no fenólicos*. Además, la estabilidad de los ligandos hexadentados se incrementa con el número de grupos donadores implicados en la coordinación del metal (denticidad). De ahí, que el isómero Fe(III)-*o,p*EDDHA tenga menor constante de estabilidad que el Fe(III)-*o,o*EDDHA.

Sin embargo, cuando un quelato se aplica a un cultivo hidropónico o en un sistema suelo/planta, existen muchas reacciones que pueden hacer variar su estabilidad intrínseca, como por ejemplo reacciones de intercambio de ligando y/o metal. Por ello, es más adecuado el cálculo del pFe y la distribución de las especies, utilizando un sistema que incluya la mayoría de los equilibrios que están presentes en las condiciones reales donde se aplica el quelato férrico y que afectan a su estabilidad. Este cálculo se realiza con ayuda de los programas de especiación química como el MINTEQA2, con el que se pueden obtener los diagramas de estabilidad de los quelatos frente al pH en diferentes condiciones (Yunta *et al.*, 2003a; Yunta *et al.*, 2003c). Con estos estudios se ha podido ver, por ejemplo, que los quelatos sintéticos *no fenólicos* como el EDTA y análogos, no tienen la estabilidad necesaria para mantener suficiente Fe en la disolución del suelo a pH superiores a 6, ya que el Fe puede ser desplazado por otros elementos como el Zn, Mn o Ca. También se observó que a concentraciones elevadas de Cu(II) ($>10^{-4}$ M), el Fe(III)-*o,p*EDDHA deja de ser estable y el Fe es desplazado por el Cu (Yunta *et al.*, 2003a).

Una característica común y preocupante de estos agentes quelantes sintéticos es su alta persistencia en el medioambiente que puede afectar a la movilidad de otros metales del suelo debido a su participación en reacciones de intercambio de metal y/o ligando, alterando el equilibrio natural de los metales en el medioambiente (Nowack, 2002). Un estudio reciente sobre el potencial redox del Fe(III)-*o,o*EDDHA elimina la posibilidad de la fotodegradación como mecanismo a la eliminación de este quelato en el medioambiente (Gómez-Gallego *et al.*, 2005). Sin embargo, varios estudios han demostrado la fotodegradación de quelatos férricos no fenólicos particularmente de EDTA y DTPA.

2.2 Aplicación de los quelatos férricos sintéticos en la agricultura

La aplicación de quelatos en la agricultura puede hacerse por cuatro vías:

- **Aplicación foliar**

La efectividad de esta aplicación depende de la formulación y el procedimiento utilizados. En general, la absorción del Fe vía foliar mejora notablemente cuando el quelato se adiciona conjuntamente con tensioactivos que mejoran la penetración del Fe en la hoja (Fernández *et al.*, 2009). Sin embargo, la respuesta de los rociados foliares de Fe sigue siendo muy variable, dependiendo principalmente de factores relacionados con la planta (especie, variedad, condiciones de crecimiento, etc.), el medioambiente (humedad relativa, temperatura, luz, etc.) y factores físico-químicos (Fernández y Ebert, 2005).

Algunos autores recomiendan el uso de aplicaciones foliares de disoluciones ácidas de quelatos para corregir la clorosis férrica (Chen y Barak, 1982; Wallace, 1988). Sin embargo, otros autores comprobaron que eran más eficaces los tratamientos foliares con sales inorgánicas de Fe ($FeSO_4$) que los de quelatos (Álvarez-Fernández *et al.*, 2004; Fernández *et al.*, 2006). Además, el elevado precio de los agentes quelantes respecto a las sales de Fe hace que la aplicación foliar de quelatos férricos no sea muy recomendable (Modaihsh, 1997). Recientemente, se ha comparado la eficacia de rociados foliares con diferentes quelatos y complejos naturales obteniéndose mejores reverdecimientos para los quelatos sintéticos y complejos de aminoácidos, mientras que la movilización del Fe hacia las raíces solo ocurrió con los lignosulfonatos de Fe (Rodríguez-Lucena *et al.*, 2010).

- **Inyección al tronco**

Este tipo de aplicación de quelatos férricos sintéticos sólo es posible en árboles y cuando su interés económico lo justifique. La inyección de quelatos férricos se lleva a cabo en forma líquida, pero otro tipo de fertilizantes de Fe ($FeSO_4$ y citrato férrico) se pueden añadir en forma sólida.

Los implantes sólidos de sales inorgánicas ($FeSO_4$) en el tronco tuvieron un efecto muy duradero, de hasta un año en peral, en la corrección de clorosis férrica (Abadía *et al.*, 2004; Larbi *et al.*, 2002). Asimismo, se comparó el efecto de inyecciones líquidas de quelatos férricos de Fe-EDDHA y Fe-EDDHMA en olivo y melocotonero encontrándose recuperaciones de la clorosis en ambos tratamientos y especies, siendo el efecto más prolongado en el caso del Fe-EDDHMA (Fernández-Escobar *et al.*, 1993).

- **Aplicación al suelo o sustrato**

Esta aplicación es, junto con la adición de quelatos a las aguas de riego, el método más común y donde la aplicación de quelatos férricos resulta más eficaz que otros productos como las sales de Fe, acidificantes y humatos (Lucena, 2003).

Los quelatos más eficaces para suelos calizos son el Fe-EDDHA y derivados, ya que el Fe-EDTA y Fe-DTPA son inestables a pH alcalino. Dentro de los quelatos férricos con grupos fenólicos en su estructura, los diferentes estudios realizados muestran resultados contradictorios. Así, algunos autores obtuvieron mayor eficacia con un producto de Fe-EDDHMA frente a uno de Fe-EDDHA en plantas de tomate crecidas en un sustrato calizo (Álvarez-Fernández *et al.*, 1996). Sin embargo, se consiguió un efecto más duradero en tratamientos con Fe-EDDHA que con Fe-EDDHMA en girasol (*Helianthus annus*), melocotonero y peral (Álvarez-Fernández *et al.*, 2005). Existen también discrepancias en cuanto a la eficacia de los isómeros de un mismo agente quelante como es el caso de los isómeros de *o,o* y *o,p*EDDHA. Así, se obtuvo mayor eficacia con el quelato Fe(III)-*o,p*EDDHA que con el Fe(III)-*o,o*EDDHA en cultivos hidropónicos de soja (*Glycine max* L.) (García-Marco *et al.*, 2006). Sin embargo, estudios en suelos calizos mostraron lo contrario, concluyendo que el isómero Fe(III)-*o,o*EDDHA es mejor fertilizante que el Fe(III)-*o,p*EDDHA (Rojas *et al*., 2008; Schenkeveld *et al*. 2008).

En solución nutritiva se ha comparado la eficacia de cinco quelatos sintéticos (Fe-EDDS, Fe-IDHA y tres formulaciones comerciales de Fe-EDTA) y diez complejos (humatos, lignosulfonatos, aminoácidos, glicoproteínas, polipéptidos, citrato y gluconato) aplicados a plantas de soja con deficiencias de Fe. Los resultados de este estudio indicaron que los quelatos sintéticos favorecían el crecimiento de las plantas en mayor medida que los complejos, así como el incremento en la concentración de Fe y en el índice SPAD. Entre los complejos, sólo la transferina mostró una eficacia similar a la de los quelatos (Rodríguez-Lucena *et al.*, 2010).

- **Adición a las aguas de riego**

Este tipo de aplicación sólo es factible en riego localizado, como es el caso de la fertirrigación. Con este tipo de regadío normalmente la clorosis se ve agravada ya que, además del uso de aguas y sustratos calizos, las raíces de la planta se desarrollan poco lo que conlleva una menor superficie de contacto con el sustrato. Este hecho hace necesario realizar un aporte regular de Fe en forma soluble en cultivos fertirrigados. Ninguna sal

inorgánica puede ser utilizada en fertirrigación ya que pueden precipitar como óxidos o fosfatos de Fe que obturarían los goteros. Por tanto, los quelatos sintéticos son los fertilizantes férricos más empleados (Lucena, 2003).

2.3 Mecanismo de acción de los quelatos férricos sintéticos

Los quelatos férricos sintéticos se aplican principalmente a plantas de Estrategia I, ya que las de Estrategia II no suelen padecer deficiencia de Fe.

El mecanismo de acción propuesto para los quelatos férricos sintéticos es igual al representado en la Figura 1-2 para plantas de Estrategia I y se compone de las siguientes etapas (Shenker y Chen, 2005):

1. Adsorción del Fe(III)-quelato en la raíz.
2. Reducción del Fe(III)-quelato a Fe(II)-quelato por la FCR.
3. Disociación del quelato y liberación del Fe(II).
4. Transporte del Fe(II) al interior de la célula a través del transportador de membrana IRT1.

La reducción obligatoria de los Fe(III)-quelatos fue descrita por primera vez en plantas de soja utilizando la formación del complejo de Fe(II) con ácido batofenantrolindisulfónico (BPDS; Chaney *et al.*, 1972). El agente reductor que se ha propuesto como donor de electrones a la enzima FCR es el NAD(P)H, siendo el Fe(III)-quelato el aceptor de electrones (Harrington y Crumbliss, 2009; Shenker y Chen, 2005). A modo de ejemplo, en la Figura 1-6 se muestran los potenciales redox de algunos compuestos de Fe a pH 7. Sin embargo, estudios recientes sobre el potencial redox de algunos quelatos como el Fe(III)-*o,o*EDDHA cuestionan esta teoría ya que este potencial se considera demasiado negativo (-497 V) para que la reacción de reducción sea termodinámicamente favorable (Gómez-Gallego *et al.*, 2005). Estos autores han propuesto que la especie aceptora de electrones desde la enzima FCR podría ser la especie protonada [Fe(III)HL] en lugar de la [Fe(III)L]$^-$.

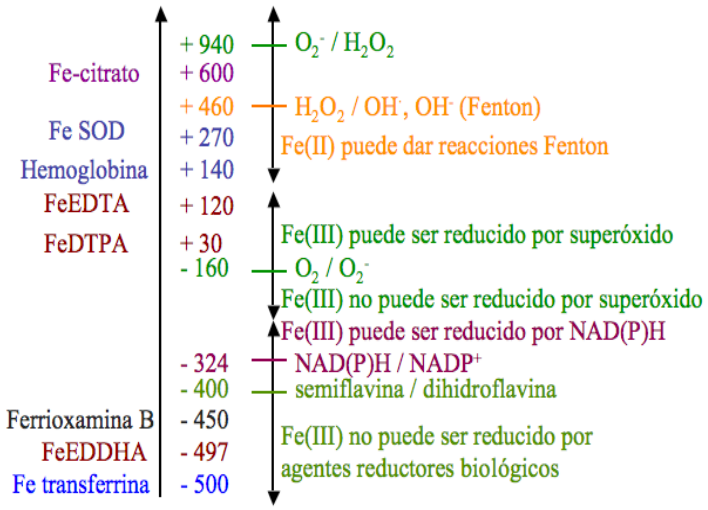

Figura 1-6. Potenciales redox de Fe(III)/Fe(II) a pH 7 (modificada de (Pierre *et al.*, 2002)).

Después de la disociación del quelato, el agente quelante queda libre en la solución del suelo, pudiendo disolver el Fe nativo del suelo y transportarlo hacia la rizosfera. Este proceso es conocido como efecto "shuttle" (Lucena, 2006). Según este mecanismo propuesto el agente quelante queda fuera de la planta y sólo el Fe(II) es incorporado al interior. Además, el Fe(II) producido por la FCR y no absorbido por la planta tiene que ser re-oxidado. Se ha propuesto que los propios agentes quelantes pueden actuar como catalizadores de la reacción de re-oxidación favoreciendo la formación de nuevo del quelato férrico (Lucena, 2006). Existen algunos trabajos que han detectado el agente quelante dentro de la planta. Así, se ha encontrado Fe(III)-EDDHA en exudados de zinia (*Zinnia elegans*), girasol y soja (Tiffin *et al.*, 1960), en hojas de tabaco (*Nicotiana tabacum*) (Jeffreys y Wallace, 1968) y en tejidos de tomate, pepino y lechuga (*Lactuca sativa*) (Bienfait *et al.*, 2004). En la Figura 1-7 se pueden ver a simple vista manchas rojas de Fe-EDDHA en hojas de tomates cloróticos tratados en hidroponía con altas concentraciones de Fe(III)-EDDHA.

Figura 1-7. Hojas de tomate clorótico tratado en hidroponía con 1000 µM Fe(III)-EDDHA.

Estudios con isótopos radiactivos, ^{55}Fe(III)-^{14}C-EDDHA (Tiffin y Brown, 1961) y ^{59}Fe(III)-^{14}C-EDDHA (Römheld y Marschner, 1981a), confirmaron la entrada de EDDHA o, al menos de un subproducto de su degradación, tanto en plantas de Estrategia I como de Estrategia II. En ambos trabajos no se encontraron diferencias en el ^{14}C tomado por plantas deficientes y suficientes en Fe, lo que indica que la entrada de agente quelante a la planta es independiente del estado nutricional de ésta. Sin embargo, la relación ^{59}Fe/^{14}C o ^{55}Fe/^{14}C en las plantas de Estrategia I fue mucho mayor en plantas deficientes en Fe que en plantas suficientes (aproximadamente valores de Fe/C de 25 y 6, respectivamente), lo que sugiere una activación del mecanismo de toma de Fe en situaciones de deficiencia. En las plantas de Estrategia II, la relación ^{59}Fe/^{14}C fue aproximadamente 1 independientemente del estado nutricional de la planta (Römheld y Marschner, 1981a).

En la Figura 1-8 se muestran las autorradiografías de plantas de soja deficientes y suficientes en Fe tratadas con ^{55}Fe-^{14}C-EDDHA. Como se puede observar, el contenido de ^{55}Fe en la planta deficiente es mucho mayor que en la suficiente, sin embargo, el contenido de ^{14}C es muy similar en ambas plantas (Tiffin y Brown, 1961).

Las características que idealmente debe tener un quelato para que su uso con fines agrícolas sea viable son las siguientes (Brown, 1969; Shenker y Chen, 2005):

El metal quelado no debe ser sustituido fácilmente por otro metal.
Debe ser estable frente a la hidrólisis.
No debe descomponerse por la acción de los microorganismos del suelo.
Debe ser soluble en agua.

No debe fijarse fácilmente a la superficie de las partículas del suelo.
Debe estar en forma accesible para la planta.
No debe ser tóxico en dosis adecuadas.
Debe estar en forma fácilmente aplicable al suelo y a la planta.
Debe ser barato.

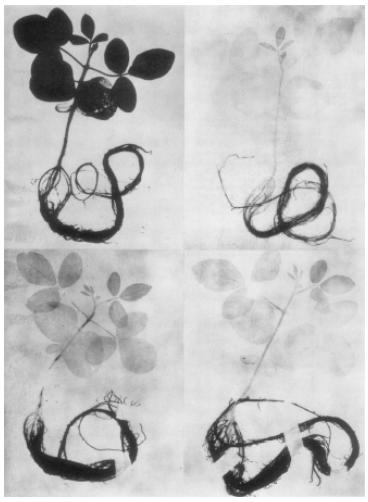

Figura 1-8. Autorradiografías de plantas de soja tratadas con ^{55}Fe-^{14}C-EDDHA. Las plantas de arriba muestran el ^{55}Fe de una planta clorótica (izquierda) y de una verde (derecha). Las plantas de abajo muestran el ^{14}C de una planta clorótica (izquierda) y de una verde (derecha); (Tiffin y Brown, 1961).

3. LA ESPECTROMETRÍA DE MASAS APLICADA AL ESTUDIO DE COMPLEJOS METÁLICOS EN PLANTAS

Los iones metálicos son fundamentales en muchos procesos biológicos. La formación de complejos con diversos ligandos puede servir para estabilizar estructuras, desencadenar mecanismos y controlar reacciones *redox*. Por ello, el interés en desarrollar técnicas que permitan identificar esos complejos metálicos y obtener información sobre su naturaleza química ha ido aumentando en los últimos años. En el campo de la fisiología y bioquímica vegetal, la identificación y cuantificación de complejos metálicos ha resultado especialmente interesante en los siguientes campos (Mounicou *et al.*, 2009):

i) La toma y biodisponibilidad de elementos esenciales, sobre todo el Fe y Zn. El principal objetivo de estos estudios radica en conocer la transferencia de estos metales a las partes comestibles de las plantas para combatir la malnutrición que afecta a más de la mitad de la población mundial, especialmente en países en desarrollo.

ii) La hiperacumulación de metales en plantas para aplicaciones de fitorremediación y fitoextracción. Algunas plantas, conocidas como hiperacumuladoras, son capaces de almacenar metales en la parte aérea a concentraciones que serían tóxicas en otras especies. La fitorremediación utiliza este tipo de plantas para favorecer la eliminación de metales pesados tóxicos que se encuentren en exceso en suelos y aguas. Por otra parte, la

fitoextracción puede resultar útil para recuperar metales preciosos utilizando el mismo tipo de plantas.

El estudio de complejos metálicos en muestras biológicas resulta muchas veces complicado debido a que la extracción del analito de su entorno original conlleva el riesgo de alterar su identidad. La espectroscopía de absorción de rayos X (XAS) es una de las técnicas más atractivas para el análisis directo de complejos metálicos en muestras biológicas, ya que proporciona información del entorno de coordinación del metal y el estado de oxidación (Lombi *et al*., 2010). Sin embrago, la elevada concentración de metal y el grado de pureza requeridos por esta técnica hace que su aplicación sea limitada a unos pocos organismos como por ejemplo las plantas hiperacumuladoras (Mounicou *et al.*, 2009). Además, esta técnica sólo puede cofirmar la presencia de especies metálicas esperadas en una muestra pero no puede identificar nuevas especies (Mounicou *et al*., 2009).

El análisis de complejos metálicos presentes a concentraciones traza se puede llevar a cabo mediante técnicas acopladas que combinen la separación con alta resolución de la cromatografía o la electroforesis con una detección sensible por espectrometría de masas (MS) elemental o molecular. La MS se basa en la ionización de la muestra y en la separación y registro, según su relación masa/carga (*m/z*), de los iones producidos. La necesidad de una separación previa al análisis por MS radica en proporcionar suficiente resolución para evitar la coelución de varias especies con el mismo elemento (en el caso de MS elemental) o la supresión de ionización del analito debido a otras sustancias fácilmente ionizables que coeluyan con él (en el caso de MS molecular). Sin embargo el uso de técnicas acopladas necesita que las especies metálicas sean químicamente inertes para que no se intercambien con otros ligandos en el proceso de análisis. Por ello hay que tomar precauciones tanto en la separación cromatográfica como en la extracción del analito de la matriz biológica.

3.1 Identificación de complejos metálicos por espectrometría de masas

Dentro de la MS molecular, el electrospray (ESI) es probablemente el método de ionización preferido para la identificación de complejos metálicos, aunque la ionización química a presión atmosférica (APCI) y la ionización por desorción láser asistida por una matriz (MALDI) son también utilizadas. La ionización por ESI se basa, fundamentalmente, en una electronebulización o electropulverización de la muestra líquida que fluye a través de un capilar al que se le aplica un potencial elevado. En la Figura 1-9 se muestra un mecanismo implicado en la formación de iones por electrospray (Cole, 1997). La principal ventaja del ESI es que el método de formación de iones es suave, lo que permite identificar incluso moléculas con alta masa molecular sin ser fragmentadas (Fenn, 2003).

La elucidación de estructuras de complejos metálicos se puede abordar mediante la comparación de la masa exacta del ión molecular precursor y las masas de los iones producto provenientes de la fragmentación producida en la espectrometría de masas en tándem (MS/MS). Para la determinación de masa exacta es importante tener equipos de alta resolución, por ejemplo aquéllos con analizador de tiempo de vuelo (TOF) o ión-ciclotrón con transformada de Fourier (FT-ICR o FTMS). Además una elevada resolución permite distinguir la huella isotópica del metal, lo cual es una herramienta muy útil en la identificación de complejos metálicos. El analizador TOF se basa en medir el tiempo que tarda un ión en atravesar una distancia determinada en el espectrómetro de masas. De esta manera los iones menos pesados (con menor *m/z*) llegan antes al detector que los de mayor

m/z. Este mecanismo requiere que los iones salgan de la fuente de ionización de forma pulsada lo que se suele conseguir focalizando los iones ortogonalmente respecto al tubo de vuelo y usando un campo eléctrico pulsado (Choi *et al*., 2003; Bristow *et al*., 2003). El analizador FT-ICR se basa en la precesión de los iones bajo un campo magnético fuerte a una determinada frecuencia característica de su *m/z*. Realizando un barrido rápido de frecuencias se obtiene una señal compleja de la que se deducen las frecuencias individuales mediante la transformada de Fourier. Posteriormente estas frecuencias se transforman en los correspondientes valores de *m/z*. Este analizador tiene muy alta resolución y sensibilidad pero un elevado coste.

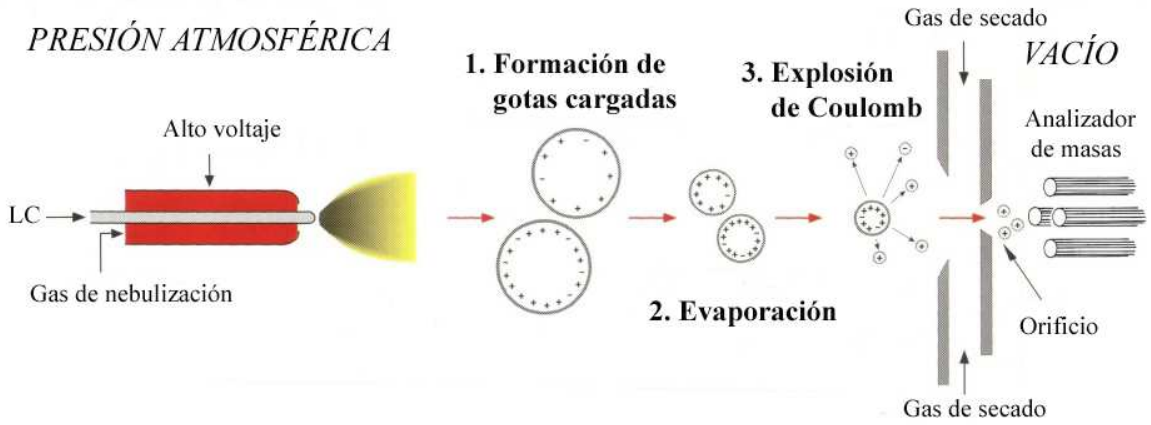

Figura 1-9. Esquema de la formación de iones por electrospray (modificada del tutorial de Applied Biosystems).

La insuficiencia de la determinación de masa exacta para la identificación de algunos compuestos en base a su fórmula empírica ha sido compensada por la espectrometría de masas en tandem (MS/MS) ya que da idea de los enlaces químicos presentes en la molécula (Mounicou *et al*., 2009). La MS/MS consiste en el acoplamiento, en el tiempo o en el espacio, de dos etapas de análisis de masas. El concepto de acoplamiento de MS/MS en el espacio es ilustrado en la Figura 1-10 e involucra dos sistemas de espectrometría de masas; el primer sistema (MS-1) lleva a cabo la selección del ión precursor o ión padre a partir de un haz de iones producido en la fuente de ionización. A continuación, en una región intermedia, se lleva a cabo la fragmentación del ión padre y en el segundo sistema de MS (MS-2) se analizan los iones producto o hijos producidos en la región intermedia.

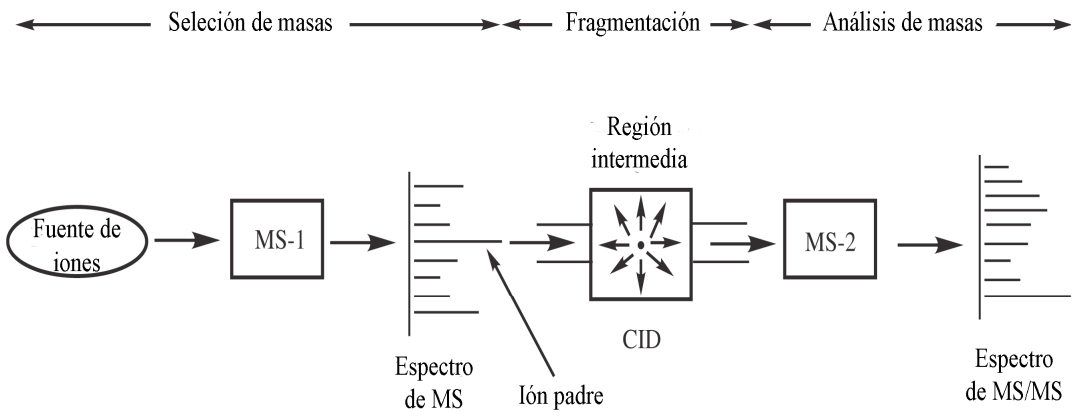

Figura 1-10. Esquema del principio básico de la espectrometría de masas en tándem. (Modificada de Dass, 2006)

La identificación de complejos metálicos se puede llevar también a cabo mediante MS elemental utilizando la fuente de ionización de plasma de acoplamiento inductivo (ICP-MS). El ICP-MS carece de especificidad molecular, por lo que se suele acoplar a técnicas de separación como el HPLC. En un ICP la muestra líquida es vaporizada e ionizada gracias a un plasma, generalmente de Ar. El plasma es sustentado por medio de campos eléctricos y magnéticos fluctuantes producidos por un generador de radiofrecuencia a través de una bobina de inducción (Houk, 1986). Las altas temperaturas alcanzadas en la fuente ICP aseguran una completa descomposición de la muestra en sus átomos constituyentes y su posterior ionización, por lo que el ICP ha sido descrito como una fuente ideal de iones en la identificación de metales (Meija *et al*., 2006). Una mejora importante sobre las técnicas existentes ha sido el reciente desarrollo, diseño y construcción de un espectrómetro de masas MS-TOF que emplea simultáneamente las fuentes de ionización ESI e ICP (Rogers *et al*., 2009, 2010a,b). La aplicación en un futuro de este tipo de instrumentos tendrá gran trascendencia en la identificación de complejos metálicos.

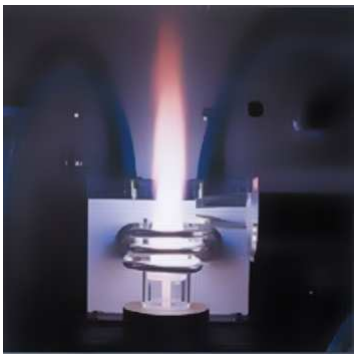

Figura 1-11. Foto de un plasma de argón (http://www.pa.ingv.it/laboratori/tracce/elementi.html)

Encontramos numerosos ejemplos en la bibliografía de identificación de complejos metálicos en plantas utilizando las técnicas de MS comentadas hasta ahora. Por ejemplo, se ha estudiado la estabilidad de los complejos de fitoquelatinas (oligopéptidos que secuestran

metales pesados) con Cd usando el isótopo ^{116}Cd y analizando los complejos por cromatografía de par iónico acoplada a ICP-MS (Loreti *et al.*, 2005). La cromatografía de exclusión por tamaño (SEC) acoplada a ICP-MS ha permitido estudiar procesos de oxidación del glutatión (GSH) con Cu^{2+}, Cd^{2+} y Pb^{2+} (Polec-Pawlak *et al.*, 2007). En este mismo trabajo, se utilizó SEC-ESI-MS para observar la formación de complejos metálicos con glutatión disulfuro (GSSG) y definir la estequiometría metal:ligando mediante el patrón isotópico de la molécula. Complejos metálicos de glutatión-Pb-EDTA se han identificado también por ESI-MS en plantas de vetiver (*Vetiveria zizanoides*) usadas para fitorremediación de suelos contaminados con Pb mediante tratamiento con EDTA (Andra *et al.*, 2009). En estudios de complejos Fe(III)-fitosideróforos analizados con espectrometría de masas con bombardeo con átomos acelerados (FAB-MS) se observó una reducción de estos complejos a Fe(II)-fitosideróforos debido al proceso de ionización (Kenny y Nomoto, 1995). Esta misma técnica junto con la resonancia magnética nuclear (NMR) ha sido utilizada para identificar dos nuevos fitosideróforos en plantas herbáceas perennes (Ueno *et al.*, 2007). El uso de un proceso de ionización más suave como el ESI-MS permitió la identificación satisfactoria de complejos Fe(III)-fitosideróforos y Zn(II), Cu(II) y Ni(II)-fitosideróforos en trigo (*Triticum aestivum*) (Xuan *et al.*, 2006). La espectrometría de MS/MS se ha utilizado para caracterizar los espectros de fragmentación de quelatos férricos de varios grupos distintos de sideróforos conocidos con el objeto de ayudar a identificar nuevos sideróforos (Majwi *et al*., 2008). En el caso de quelatos férricos sintéticos utilizados como fertilizantes, en 1998 se demostró que la ESI-MS era una herramienta muy útil en la identificación de complejos metálicos de EDTA, incluyendo el Fe-EDTA, utilizando la huella isotópica característica de cada metal (Baron y Hering, 1998). Más tarde se utilizó la misma técnica para la caracterización de fertilizantes férricos de EDTA, DTPA, EDDHA, EDDHMA, EDDHSA y EDDCHA (Cantera *et al.*, 2002).

3.2 Cuantificación de complejos metálicos por espetrometría de masas

El metal presente en los complejos metálicos es frecuentemente usado como diana para la su cuantificación mediante ICP-MS, debido a su especificidad isotópica, elevada sensibilidad independientemente de la matriz de la muestra, alto rango dinámico y fácil acoplamiento con técnicas de separación (Mounicou *et al*., 2009; Meija *et al*., 2006). Por ejemplo, se ha utilizado ICP-MS acoplado a cromatografía de fase reversa para la determinación de complejos Cd-fitoquelatina (Cd-PC2, Cd-PC3 y Cd-PC4) en raíces y parte aérea de Arabidopsis obteniéndose límites de detección del orden de 50-100 ng l^{-1} (Sadi *et al.*, 2008). La misma técnica pero acoplada a cromatografía de intercambio aniónico se utilizó para cuantificar complejos de Co(II)-, Ni(II)-, Cu(II)-, Fe(III)-fitosideróforos (mugineico y desoximugineico) con límites de detección del orden de 0.1-2.8 pmoles (Bakkaus *et al.*, 2006).

La cuantificación de quelatos férricos sintéticos usados como fertilizantes se ha llevado a cabo tradicionalmente mediante la técnica de HPLC-UV-vis (Barak y Chen, 1987; Deacon *et al.*, 1994; Hernández-Apaolaza *et al.*, 1997; Jen y Chen, 1992; Lucena *et al.*, 1996; Vande Gucht, 1994). Sin embargo, recientemente, la MS está adquiriendo relevancia en este campo como son las determinaciones de agentes quelantes derivados de los ácidos poliaminocarboxílicos (por ej. EDTA, CDTA, DTPA) por HPLC-ICP-MS llegando a límites de detección del orden del nanomolar (Ammann, 2002). La MS molecular también tiene aplicaciones en el campo de la cuantificación de quelatos metálicos. Así pues, encontramos determinaciones de complejos de EDTA con metales (Cu, Pb, Cd, Al y Fe(III)) por ESI-MS con límites de detección entre 1-2 µM (Baron y Hering, 1998). Para el mismo quelato pero con un método de cromatografía iónica acoplada a ESI-MS, se

consiguió su cuantificación con diferentes metales como el Al, Cd, Cu, Co, Mn, Ni, Pb y Zn en solución de suelo y savia de xilema, con límites de detección del orden de 0.1-1 µM (Collins *et al.*, 2001). Igualmente, se pudo determinar Fe(III)-EDTA en aguas industriales y residuales mediante cromatografía de fase reversa acoplada a ESI-MS con un límite de detección de 1 µg L^{-1} (Dodi y Monnier, 2004).

Recientemente, el desarrollo de métodos analíticos que combinan ESI-MS e ICP-MS esta adquiriendo especial interés ya que combinan la especificidad molecular de la ESI-MS para la identificación inequívoca de los complejos metálicos y la elevada sensibilidad del ICP-MS para la cuantificación, obteniendo límites de detección muy bajos. Ejemplos de esta combinación de técnicas de MS es la determinación de complejos de Zn-aminopolicarboxilatos (HEDTA, NTA, EDTA y DTPA) en soluciones de suelo (Chen *et al.*, 2009), la identificación y cuantificación de nuevas especies de As-fitoquelatinas en raíces, savia de xilema, tallos y hojas de girasol (Raab *et al.*, 2005), la determinación de Cd y Pb-fitoquelatinas en guisante (Baralkiewicz *et al.*, 2009) y la reciente determinación de especies de Fe-citrato en savia de xilema de tomate (Rellán-Álvarez *et al.*, 2010). Como ya se ha comentado se están diseñando y construyendo espectrómetros de masas que combinan ambas fuentes de ionización ESI e ICP (Rogers et *al.*, 2009, 2010a,b) lo que supondrá un gran avance en este tipo de análisis.

Los métodos de cuantificación clásicos en MS son el de patrón externo y el de estándar interno. El método de patrón externo requiere disponer de los patrones de las sustancias que se quieren cuantificar y analizarlos de forma separada a la muestra. El procedimiento a seguir consiste en hacer una curva de calibrado a diferentes concentraciones de patrón y ajustar los valores obtenidos generalmente a una recta mediante mínimos cuadrados (Skoog *et al.*, 2008). El método de estándar interno requiere disponer, además de los patrones de las sustancias que se quieren cuantificar, de un estándar interno que se añada a la muestra y a los patrones de la curva de calibrado en cantidad conocida. Un estándar interno tiene que cumplir idealmente las siguientes condiciones (Wieling, 2002):

no estar presente en la muestra,

no reaccionar con ninguno de los componentes de la muestra,

ser estable en las condiciones de análisis y

ser de naturaleza parecida a la de los analitos.

La recta de calibrado es del tipo:

$$A_i \frac{C_{SI}}{A_{SI}} = aC_i + b$$

donde A_i es el área o intensidad de señal de los patrones, A_{SI} el área o intensidad del estándar interno, C_{SI} es la concentración del estándar interno y C_i la concentración del analito. Este procedimiento es el que consigue mayor precisión y exactitud, ya que compensa los errores en la inyección y las variaciones en las condiciones experimentales (por ejemplo fluctuaciones del proceso de electrospray). Como inconveniente puede destacarse que la elección del estándar interno adecuado no es una tarea sencilla. Generalmente se suele utilizar como IS la molécula marcada con isótopos estables como el D, ^{13}C, ^{15}N, etc. Sin embargo, el uso e IS no siempre asegura una respuesta constante de la relación analito/IS ya que los componentes de la matriz pueden afectar de diferente manera

a la ionización del analito y del IS (Taylor, 2005) o pueden aparecer reacciones de intercambio entre isótopos, sobre todo el IS marcados con deuterio (Chavez-Eng *et al.*, 2002). Además, algunos autores han demostrado recientemente la importancia de evaluar la pureza del estándar interno antes de su utilización (Bergeron *et al*., 2009)

Recientemente ha adquirido especial relevancia en la cuantificación por MS el análisis por dilución isotópica (DI; Rodríguez-González *et al*., 2005; Schaumlöffel y Lobinski, 2005; Rodríguez-González y Carcía-Alonso 2010). Esta técnica analítica consiste en medir las relaciones isotópicas de muestras que han sido alteradas en su composición por la adición de una cantidad conocida de un trazador enriquecido isotópicamente. El aspecto novedoso de la dilución isotópica es el uso de los isótopos para cuantificar. Este hecho le otorga una serie de ventajas únicas con respecto a las otras formas de cuantificar y le permiten proporcionar medidas de referencia. La principal ventaja de la DI es que no existe "factor de sensibilidad" ya que, al usar siempre medidas relativas, compensa este efecto y en la mayoría de los casos, no se observa en absoluto. Además, las relaciones isotópicas pueden ser medidas de forma muy reproducible lo que resulta en una muy pequeña incertidumbre en el análisis. Esta excelente precisión surge del hecho de que un isótopo de un elemento es el estándar interno ideal de otro isótopo de ese elemento ya que va a tener el mismo comportamiento físico y químico. Por ello, la dilución isotópica corrige perfectamente estos efectos de deriva e inestabilidades del equipo y la mayoría de los efectos de matriz.

Para cuantificar con DI sólo es necesario conocer con exactitud las abundancias relativas de todos los isótopos de un elemento (por ej. ^{54}Fe, ^{56}Fe, ^{57}Fe y ^{58}Fe) y la concentración del mismo en el trazador mediante el correspondiente certificado o mediante un análisis por dilución isotópica inversa con una disolución de patrón natural de concentración conocida. La ecuación de la dilución isotópica se ha expresado de diferentes formas en la bibliografía, sin embargo para el presente trabajo se ha empleado la siguiente ecuación (Rodríguez-González *et al.*, 2005):

$$C_S = C_{Sp} \frac{W_{Sp}}{W_S} \frac{Aw_S}{Aw_{Sp}} \frac{A^b{}_{Sp}}{A^a{}_S} \left(\frac{R_m - R_{Sp}}{1 - R_m R_S} \right)$$

donde C_s es la concentración desconocida del elemento en la muestra (s), C_{sp} es la concentración del elemento en la disolución isotópica enriquecida o trazador isotópico (sp), W_s y W_{sp} son los pesos tomados de muestra y trazador respectivamente, Aw_s y Aw_{sp} son los pesos atómicos del elemento en la muestra y en el trazador, $A^b{}_{sp}$ es la abundancia isotópica en átomos por ciento (At%) del isótopo referencia en el trazador, $A^a{}_s$ es la abundancia isotópica (At%) del isótopo referencia en la muestra, R_M y R_{Sp} son las relaciones isotópicas (isótopo a/isótopo b) en la mezcla y en el trazador, respectivamente, R_S es la relación isotópica (isótopo b/isótopo a) en la muestra. En el caso del análisis de elementos con abundancias naturales sólo se necesita determinar experimentalmente R_m.

Existe una herramienta matemática que simplifica bastante los cálculos de la DI y que se denomina deconvolución de perfiles isotópicos (IPD). Se entiende por perfil isotópico al conjunto de abundancias isotópicas relativas de todos los isótopos estables de un elemento (García-Alonso y Rodríguez-González, *en prensa*). El perfil isotópico de un elemento o compuesto que se encuentra en la naturaleza, y por tanto en la mayoría de las muestras a analizar, se denomina perfil isotópico natural y se puede considerar constante e invariable en toda la Tierra. Cuando se trata de un elemento o compuesto enriquecido isotópicamente,

se habla de un perfil isotópico alterado donde la abundancia relativa de uno o varios isótopos estables de dicho elemento o compuesto es claramente distinta a la natural. Cuando se realiza un análisis por dilución isotópica se utilizan al menos dos perfiles isotópicos distintos: un perfil isotópico natural y otro/s alterado/s. Uno de los perfiles isotópicos alterados (llamado trazador) es el que se añade a la muestra en una cantidad conocida para poder cuantificar el perfil isotópico natural. Si además la muestra fue tratada previamente con un perfil isotópico alterado distinto del trazador y del que, por tanto, se desconoce su concentración (por ej. un tratamiento de plantas con Fe enriquecido en un isótopo), el trazador permitirá cuantificar también la concentración de ese perfil alterado en la muestra. El cálculo matemático del IPD va por tanto encaminado a determinar la contribución de cada perfil isotópico, natural o alterado, al perfil observado en la mezcla mediante MS. Para ello se miden las relaciones isotópicas de cada isótopo respecto del más abundante y con ello se calculan las abundancias isotópicas y fracciones molares de cada perfil en la muestra (Rodríguez-Castrillón *et al.*, 2008a; Rodríguez-Castrillón *et al.*, 2008b). Como la cantidad añadida del trazador se conoce es posible determinar directamente la cantidad del elemento natural y de elemento alterado si lo hubiera.

El uso de la DI en la determinación de la concentración total de un elemento ha sido bien documentada en numerosos trabajos a lo largo de los últimos años (Fassett y Paulsen, 1989; Mann *et al.*, 2003; Monperrus *et al.*, 2004; Yip *et al.*, 2008). En el caso del Fe existen también algunos trabajos que utilizan esta técnica de dilución isotópica para su determinación en plantas (Chu *et al*., 2006; Rodríguez-Castrillón *et al.*, 2008b; Sah y Brown, 1997; Rellán-Álvarez *et al*. 2010) y en otras matrices (Busto *et al.*, 2006; Petrov y Quetel, 2005; Takaku *et al.*, 2004; Wu, 2007). Varios ejemplos de la aplicación de la técnica de dilución isotópica a la cuantificación de complejos metálicos en muestras biológicas (incluyendo muetras vegetales) se pueden en encontrar en la reciente revisión de Rodríguez-González y García-Alonso (2010).

3.3 Especiación de complejos metálicos por espectrometría de masas

Se sabe que las propiedades beneficiosas o tóxicas de un elemento están a menudo relacionadas con la especie química en la que se encuentra. Como consecuencia, la determinación de la concentración total de un elemento no resulta adecuada para medir su impacto en el medioambiente, biodisponibilidad o toxicidad (Stewart, 1999), sino que es necesario determinar las especies químicas presentes de ese elemento. Lo mismo ocurre con los complejos metálicos, siendo cada vez más relevante conocer el estado de oxidación del metal y la estequiometría metal:ligando del complejo.

El interés por desarrollar la ESI-MS como herramienta para estudiar la especiación de complejos metálicos apareció con los primeras publicaciones que demostraban la conservación del estado de oxidación de algunos iones metálicos (Blades *et al.*, 1990; Katta *et al.*, 1990) y de la forma molecular de especies lábiles (Siu *et al.*, 1989). Sin embargo, no es raro encontrar reacciones *redox* en los procesos de formación del electrospray de algunas moléculas, siendo la reducción del estado de oxidación más probable en el modo de ionización negativo (Rellán-Álvarez *et al.*, 2008). El uso de la MS/MS con analizador de cuadrupolo-tiempo de vuelo (Q-TOF) permitió observar diferentes estequiometrías metal:ligando en la especiación de Ni con malato (Ni-malato$_2$), citrato (Ni-citrato$_2$, Ni$_2$-citrato$_2$), histidina (Ni-histidina$_2$), EDTA (Ni-EDTA) y nicotianamina (Ni-nicotianamina) en extractos de tejido vegetal (Ouerdane *et al.*, 2006). Utilizando un analizador de masas de mayor resolución como el de ión ciclotrón con transformada de Fourier y la fuente de

ionización nano-ESI se consiguieron identificar especies de Fe-nicotianamina (Fe-NA) y Fe-desoximugineico (Fe-DMA) en diferentes estados de oxidación (Fe(II) y Fe(III)). Además se comprobó en este estudio que la fuente nano-ESI no alteraba el estado *redox* de las especies (Weber *et al.*, 2006). En el campo de las fitoquelatinas, estudios de ESI-MS-TOF con diferentes genotipos de cebada tolerantes al Cd demostraron que las diferentes especies de Cd-fitoquelatinas presentes en la planta eran importantes para determinar su tolerancia o no al Cd (Persson *et al.*, 2006).

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Capítulo 2

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ANALYTICAL TECHNOLOGIES TO STUDY THE BIOLOGICAL AND ENVIRONMENTAL IMPLICATIONS OF Fe-FERTILISATION USING SYNTHETIC FERRIC CHELATES: THE CASE OF Fe(III)-EDDHA – A REVIEW

Irene Orera, Javier Abadía, Anunciación Abadía and Ana Álvarez-Fernández

Plant Nutrition Department, Estación Experimental de Aula Dei, Consejo Superior de Investigaciones Científicas (CSIC), Apdo. 202, 50080 Zaragoza, Spain.

ANALYTICAL TECHNOLOGIES TO STUDY THE BIOLOGICAL AND ENVIRONMENTAL IMPLICATIONS OF Fe-FERTILISATION USING SYNTHETIC FERRIC CHELATES: THE CASE OF Fe(III)-EDDHA – A REVIEW

Abstract: The most commonly used and efficient compound for iron (Fe)-fertilisation of fruit crops grown in calcareous soils is the synthetic Fe(III)-chelate of ethylenediamine-N,N′-di-(*ortho*-hydroxyphenyl) acetic acid, usually known as Fe(III)-*o,o*EDDHA. However, the mechanism(s) of plant Fe uptake from this compound, and the environmental implications of its use, are still not completely understood. This lack of information is due, in part, to the lack of suitable analytical methods capable of determining the very low concentrations of this Fe(III)-chelate which may occur in complex matrices such as plant tissues and fluids after Fe-fertilisation. In this report, the main issues in studies of the biological and environmental implications of fertilisation with synthetic Fe(III)-chelates are discussed, focusing on new possibilities offered by recently developed analytical technologies.

Despite of the relatively low Fe requirements of plants and the high abundance of Fe in soils, Fe deficiency is a nutritional disorder that limits crop yields in many agricultural areas of the World. Fruit tree crops such as peach, pear, kiwifruit, apricot, plum, cherry, and avocado are sensitive to shortages of Fe. The cause of Fe deficiency is generally a combination of limited Fe bio-availability in the soil, which occurs particularly in calcareous and alkaline soils, and the use of susceptible genotypes that have insufficient activation of one or more Fe deficiency defence mechanisms. Iron deficiency has an important economical impact on the fruit sector because it can reduce fruit yield and quality (Álvarez-Fernández *et al.*, 2006), and also because Fe-fertilisation is expensive (200 - 400 € ha^{-1} every year; Rombolà and Tagliavini, 2006).

Iron fertilisation is the best and most commonly used technique to correct Fe deficiency in established fruit tree orchards. The active ingredients can be either inorganic or organic Fe-containing compounds. Foliar fertilisation with inorganic Fe compounds (e.g., $FeSO_4$) or some organic Fe complexes, including natural (e.g., citrate) and synthetic ligands such as ethylenediamine tetraacetic acid (Fe(III)-EDTA; Figure 2-1-**1**), N-(2-hydroxyethyl)ethylenediaminetriacetic acid (Fe(III)-HEDTA; Figure 2-1-**2**) and di-ethylenetriamine pentaacetic acid (Fe(III)-DTPA; Figure 2-1-**3**), could alleviate Fe-deficiency although this method is still not very common (Abadía *et al*., 2004). Trunk injection of liquid Fe fertilisers, or solid branch implants of Fe compounds are still less frequent, in spite of the long-lasting efficiency that can be obtained with one application per year (Abadía *et al*., 2004). The most widely used Fe-fertilisation technique for fruit crops grown in calcareous soils is an annual soil application of expensive synthetic Fe(III)-chelates such as ethylenediamine-N,N′-di-(*ortho*-hydroxyphenyl) acetic acid (Fe(III)-*o,o*-EDDHA; Figure 2-1-**4**) and analogues such as ethylenediamine-N-(*ortho*-hydroxyphenylacetic)-N'-(*para*-hydroxyphenyl-acetic) acid (Fe(III)-*o,p*-EDDHA; Figure 2-1-**5**), ethylenediamine-N-N'bis(2-hydroxy-4-methylphenylacetic) acid (Fe(III)-*o,o*-EDDHMA; Figure 2-1-**6**), ethylenediamine-N-(2-hydroxy-4-methylphenylacetic)-N'-(4-hydroxy-2-methylphenylacetic) acid (Fe(III)-*o,p*-EDDHMA; Figure 2-1-**7**), ethylenediamine-N-N'bis(5-carboxy-2-hydroxyphenylacetic) acid (Fe(III)-EDDCHA; Figure 2-1-**8**), and ethylenediamine-N-N'bis(2-hydroxy-5-sulfophenylacetic) acid (Fe(III)-EDDSHA; Figure 2-1-**9**) (Lucena, 2006).

The number of Fe fertilisers available in Spain, the largest European market for Fe fertilisers, increased significantly from 1990 to 2005 (García-Marco, 2005), although it decreased in 2007 (Figure 2-2). Approximately 80% of these fertilisers contained synthetic Fe(III)-chelates, and products containing Fe(III)-*o,o*-EDDHA as the active ingredient accounted for 56-79% of the total (Figure 2-2). Other analogues of Fe(III)-*o,o*-EDDHA accounted for a further 6-10% of the market in the same time period.

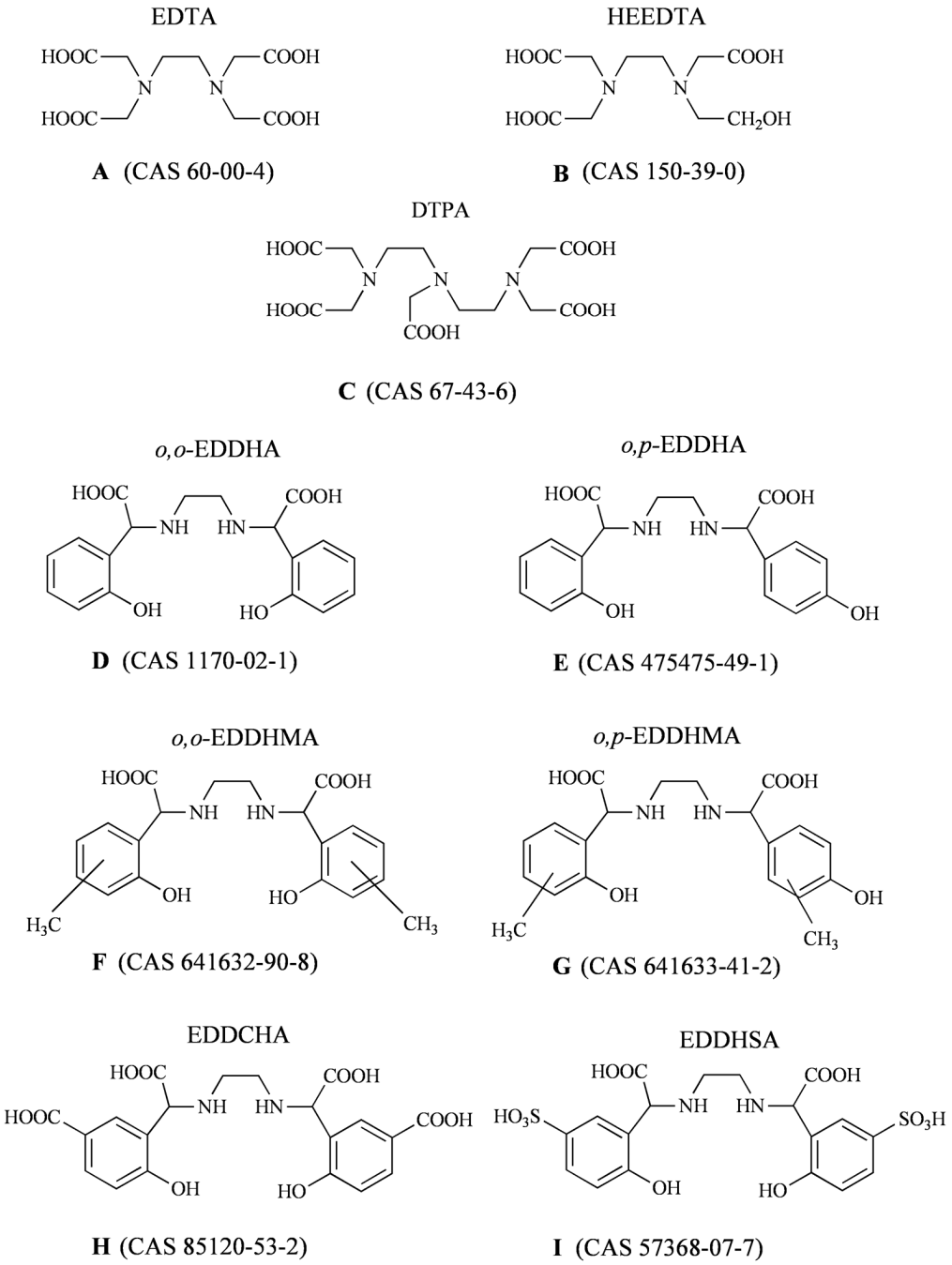

Figure 2-1. Chemical structures and abbreviated names of chelating agents allowed by current EU Commission Regulation Nº 162/2007 in Fe-fertilisers (Anon, 2007). CAS Numbers of the compounds are indicated below the formulae.

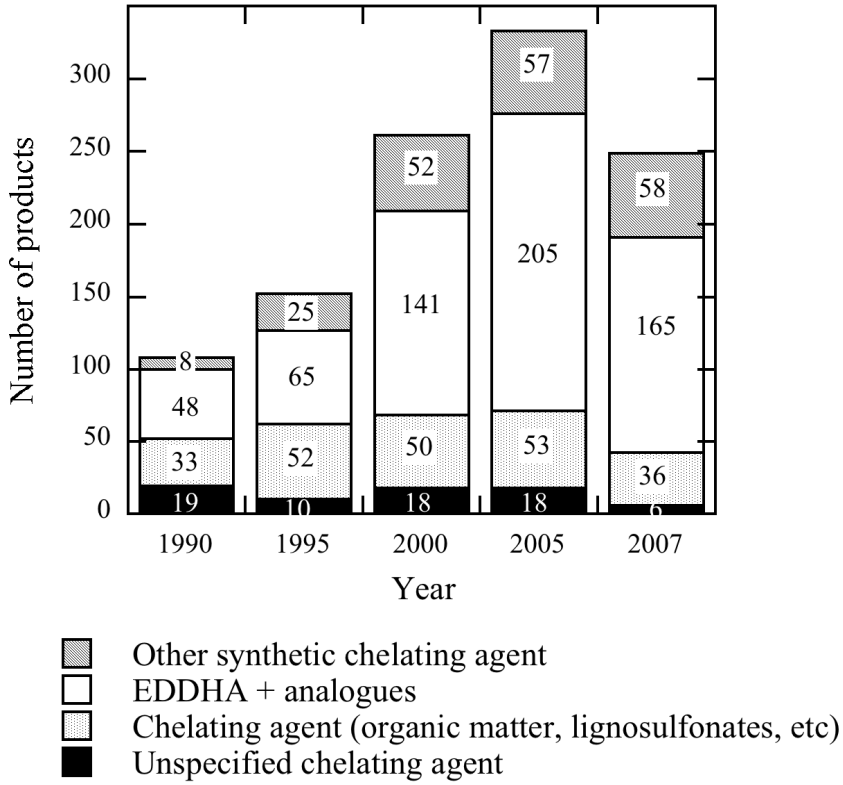

Figure 2-2. Number of Fe-fertilisers commercialised in Spain to correct Fe deficiency over the last 17 years. Data corresponding to 1990-2005 were taken from García-Marco (2005).

Despite the widespread use of these xenobiotic products, the biological and environmental implications of this practice are still not fully known. This is partially due to a lack of analytical methods capable of determining the low concentrations of synthetic Fe(III)-chelates present in environmental matrices. The organic component of synthetic Fe(III)-chelates, the amino polycarboxylate chelating agents, are also under scrutiny due to their possible effects on metal availability and mobility, because, once released, they can remain in the environment for a long time (Nowack, 2002). Synthetic Fe(III)-chelates can be involved in ligand and metal exchange reactions in plant-soil systems, which could affect the chemical behaviour and bioavailability of both Fe and the synthetic chelating agent. Therefore, to assess the effects of Fe-fertilisation with synthetic Fe(III)-chelates, the identification and quantification of all chemical forms of chelating agents are crucial steps.

This manuscript reviews the analytical techniques currently used to examine the biological and environmental implications of Fe-fertilisation, with special emphasis on Fe(III)-*o,o*-EDDHA. The limitations of the analytical techniques applied so far, as well as the improvements introduced through recent analytical advances, are discussed, and some relevant new experimental data are shown.

QUALITY OF SYNTHETIC Fe(III)-CHELATES

Commercial synthetic Fe(III)-chelate fertilisers are obtained by first synthesising the chelating agent, then incorporating Fe from an inorganic salt. The amount of synthetic Fe(III)-chelate in commercial formulations may be considered the main quality parameter. Several analytical techniques have been used to determine this value: paper, gel or thin-

layer chromatography, electrophoresis, gas chromatography and high performance liquid chromatography (HPLC) have been used as separation techniques, combined to UV/VIS or atomic absorption spectroscopy (AAS) as detection techniques (see references in Álvarez-Fernández *et al.*, 2007). Many of these methods were developed focussing on only one or a few synthetic chelates [mainly Fe(III)-EDTA]. Recently, more selective and sensitive analytical techniques such as inductively-coupled mass spectrometry (ICP/MS) and electrospray mass spectrometry (ESI/MS) coupled to HPLC have been developed to determine, simultaneously, the levels of most commercial synthetic Fe(III)-chelates in environmental matrices (Álvarez-Fernández *et al.*, 2007).

The quality of synthetic commercial Fe(III)-chelate fertilisers in Europe is tightly regulated. A group of specific parameters, including water-soluble Fe content, total chelated Fe content, and Fe content chelated by each authorised chelating agent, were recently established by the EU Regulation EC Nº 2003/2003 (Anon, 2003), later modified by EU Regulations Nº 2076/2004 (Anon, 2004), and Nº 162/2007 (Anon, 2007). Authorised chelating agents are listed in the latest modification of the EU Regulation Nº 162/2007 (Anon, 2007), which includes the corresponding CAS (Chemical Abstracts Service, American Chemical Society) Numbers, to avoid any ambiguous molecular description. The molecular structures of all authorised chelating agents are shown in Figure 2-1, with the exception of the EDDHSA condensation products (CAS Number 642045-40-7). According to the EU Regulations, the minimum permitted content of water-soluble Fe is 5% (w/w), at least 80% of the water-soluble Fe must be chelated, and 50% of the water-soluble Fe must be chelated by authorised chelating agents. In addition, fertiliser labels must indicate the Fe contents described above, the authorised chelating agents in the product when they chelate ≥1% of the water-soluble Fe, and the pH range that guarantees an acceptable stability of the chelated fraction of the Fe.

Some Official Analytical Methods have recently been approved to determine the parameters required by EU regulations. The EU Standard EN 13366 (Anon, 2001a) describes the determination of total chelated Fe by AAS or ICP after separation of chelated Fe (anionic or neutral molecules) from non-chelated Fe (cations) in a cation exchange column. This method has been recently compared unfavourably with an AOAC modified method based on the precipitation of inorganic forms at pH 9 (Villén *et al.*, 2007). Different Official methods (EU Standards, EN) have been developed to determine authorised chelates using HPLC coupled to UV/VIS spectrophotometry. These methods use anion exchange chromatography for EDTA, DTPA, HEEDTA (EN 13368-1; Anon 2001b), *o,o*-EDDHA and *o,o*-EDDHMA (EN 13368-2; Anon, 2001c), ion-pair reverse-phased chromatography for EDDHSA (CEN/TS 15451; Anon, 2006a) and reverse-phased chromatography for *o,p*-EDDHA (CEN/TS 15452; Anon, 2006b). The lack of an unique official method for the simultaneous determination of the authorized Fe(III)-chelates is due to the low specificity of the detection technique (UV/VIS), that makes mandatory a very good chromatographic separation, specially for compounds with similar molecular structure. The approval of EU Official methods led to significant changes in the Spanish Fe(III)-chelate fertiliser market, since the amount of chelated Fe in commercial fertilisers containing Fe(III)-*o,o*-EDDHA and analogues increased from 2-4% in 1999 (80 analysed products) to 3-6% in 2003-2004 (121 analysed products) (García-Marco, 2005).

The quality of commercial fertilisers is also affected by the presence of impurities coming from the chelating agent synthesis or the starting reagents. Although the first procedures to synthesize *o,o*-EDDHA and analogues produced very pure compounds, the prevalent synthesis processes in the industry lead to products which contain often by-

products (Lucena, 2006). For instance, in commercial fertilisers containing Fe(III)-*o,o*-EDDHA and Fe(III)-*o,o*-EDDHMA, *o,o*-EDDHA condensation products and positional isomers of the *o,o*-EDDHA and *o,o*-EDDHMA (e.g., *o,p*-EDDHA) have been identified by one- and two dimensional nuclear magnetic resonance (1D- and 2D-NMR) (Cremonini *et al.*, 2001; Álvarez-Fernández *et al.*, 2002), HPLC-UV/VIS (Gómez-Gallego *et al.*, 2002) and ESI/MS (Hernández-Apaolaza *et al.*, 2006). In commercial fertilisers containing Fe(III)-EDDHSA and Fe(III)-EDDCHA, unreacted starting reagents such as *p*-hydoxybenzenesulfonic (8%) and *p*-hydroxybenzoic acids (1.4-12.5%) were also found using HPLC-UV/VIS (Álvarez-Fernández *et al.*, 2002).

EFFICIENCY OF SYNTHETIC Fe(III)-CHELATES

The efficiency of a synthetic Fe(III)-chelate depends on the stability and persistence in the soil and nutrient solutions, as well as on the ability to supply Fe to plants (Lucena, 2006). Stability and persistence may be diminished in the soil solution by (i) replacement of Fe by competing metals, (ii) sorption of the Fe(III)-chelate onto soil surfaces, (iii) degradation of the Fe(III)-chelate or chelating agent, and (iv) lixiviation from the root-soil system. These processes have been studied by means of Fe speciation in soil solution using *in silico* calculations and also by direct determination of Fe(III)-chelates. *In silico* studies try to predict the Fe bound to the chelating agent, based on soil solution pH, nutrient concentrations, and protonation and stability constants of the chelates with Fe and other metals. The stability constants for most synthetic Fe(III)-chelates have been calculated using spectrophotometric data after base titration with NaOH (Yunta *et al.*, 2003), whereas stability constants for many metal chelates have not been determined yet. *In silico* calculations can be put in question by the existence of kinetically slow metal-exchange reactions that would result in a non-equilibrium speciation, and also by the presence of naturally occurring ligands that could compete with the chelating agents for available metals (Nowack, 2002). *In silico* data predict substitution of Fe by Zn, Mn, or Ca in the case of Fe(III)-EDTA at pH values above 6, but not in the case of Fe(III)-*o,o*EDDHA (Lucena, 2006). This is in agreement with the high efficacy of Fe(III)-*o,o*EDDHA in calcareous soils.

Studies using direct determinations in soil solutions usually measure Fe(III)-chelate by UV/VIS, with or without a previous chromatographic separation, and Fe by AAS and ICP. Differences concerning the concentrations of soluble Fe and Fe(III)-chelate in soil solutions and the extent of Fe(III)-chelate sorption onto soil surfaces after fertilisation were found between synthetic Fe(III)-chelates (even among isomers) depending on soil type. For example, Fe(III)-chelate efficiencies were EDDHSA > EDDCHA > EDDHA > EDDHMA > EDTA ≈ DTPA in a 60 day-study (García-Mina *et al.*, 2003), and *racemic-o,o*-EDDHA > *meso-o,o*-EDDHA > EDDHA byproducts > *o,p*-EDDHA in a 42 day-study (Schenkeveld *et al.*, 2007). Among Fe(III)-*o,o*-EDDHA isomers, the sorption on highly reactive soil materials was higher for the *meso* isomer than for the *racemic* one on ferrihydrite and organic matter, whereas for the sorption on Ca-montmorillonite no differences were observed between both isomers (Hernández-Apaolaza *et al.*, 2001). Other studies applied ^{59}Fe-labelled Fe(III)-chelates to the soil, and showed that metal isotope exchange between the ^{59}Fe bound to the chelating agent and the natural soil ^{56}Fe did occur (Hill-Cottingham and Lloyd-Jones, 1958). The degradation of Fe(III)-chelates and chelating agents has received so far little attention and it is dealt with in the environmental implications section.

The ability of Fe(III)-chelates to supply Fe to Fe-deficient plants is probably the most studied aspect related to efficiency. Leaf chlorophyll content is the method better suited to

assess the plant Fe status (Abadía *et al*., 2004). However, other parameters such as plant biomass and Fe concentrations in leaves are also widely used to evaluate the efficiency of Fe fertilisers. These parameters do not provide an unequivocal assessment of the Fe plant status. In some cases, similar leaf Fe contents have been found in Fe-deficient and Fe-sufficient leaves, and this is called the "chlorosis paradox" (Morales *et al*., 1998). Biomass and chlorophyll concentrations can be also affected by other biotic or abiotic stresses. The time dependence of the re-greening of Fe-chlorotic leaves is a parameter often used to assess fertiliser efficiency. Leaf re-greening determination is traditionally based on UV/VIS chlorophyll analysis in organic solvent leaf extracts, although this is being substituted by other methods that use immediate, non-destructive, *in vivo* estimations (SPAD; Soil Plant Analyzer Development device, from Konica-Minolta) based on the transmittance of leaves at two different wavelengths (Abadía et al., 2004). Most of the studies are aimed to compare the efficiency of one or several synthetic Fe(III)-chelates with other Fe fertilisers, considering different plant species, application times and localization of the application. In recent years, the comparison among Fe(III)-EDDHA and analogues as Fe deficiency correctors has received special attention. Commercial Fe(III)-*o,o*-EDDHA and Fe(III)-*o,o*-EDDHMA had a similar efficacy to re-green Fe-deficient plants (sunflower, peach and pear trees), both in a soil-less system and in field conditions, whereas a longer lasting effect was found with Fe(III)-*o,o*-EDDHA than with Fe(III)-*o,o*-EDDHMA (Álvarez-Fernández *et al.*, 2005). These experiments also indicated that Fe(III)-EDDHSA is a promising Fe fertiliser. In studies with tomato and pepper plants, it was found that the concentration of the *meso* isomer of Fe(III)-*o,o*-EDDHA (the lowest stability isomer) in the nutrient solution decreased more than that of the *racemic* isomer (Cerdán *et al.* 2006).

When a commercial synthetic Fe(III)-chelate is used, efficiency depends also on the commercial formulation quality. For example, in soybean plants the ferric by-products found in Fe(III)-EDDHA commercial fertilisers have less long-lasting effects than Fe(III)-*o,o*-EDDHA, as well as less fast-action effects than Fe(III)-*o,p*-EDDHA (Hernández-Apaolaza *et al*., 2006).

MECHANISMS OF ACTION OF SYNTHETIC Fe(III)-CHELATES

Plants take up Fe in the form of Fe(II) ion *via* protein transporters (Hall and Guerinot, 2006), whereas Fe in soils is in low-soluble Fe(III) forms. Therefore, in order to acquire Fe, all plants except the Poaceae family, reduce Fe(III) to Fe(II) via a plasma membrane, ferric reductase enzyme (FCR). The obligatory reduction of Fe(III)-chelates was first shown with soybean, using the formation of the Fe(II) complex with bathophenanthrolinedisulfonic acid (BPDS) (Chaney *et al.*, 1972). The same methodology was used to investigate the role of Fe(III)-*o,o*-EDDHA and analogues as substrates of the FCR enzyme in Fe-deficient cucumber plants (Lucena and Chaney, 2006). Authors concluded that in the presence of a Fe(II)-chelator, the higher the stability of the Fe(III)-chelate the lower the FCR reduction rate, even when diastereoisomers were compared (i.e., the rate of reduction of *meso* Fe(III)-*o,o*-EDDHA isomer was larger than that of the *racemic* isomer).

After the reduction of Fe(III) by the FCR, the chelating agent remains in the soil solution, thus being able to dissolve soil native Fe and transport it again to the plant rhizosphere. However, some studies have shown that whole Fe(III)-chelates or synthetic chelating agents can be also taken up by the plant. Iron(III)-EDDHA was found in zinnia, sunflower and soybean exudates (Tiffin *et al.*, 1960) and in tobacco leaves (Jeffreys and Wallace, 1968) using UV/VIS. More recently, Fe(III)-*o,o*-EDDHA was found in tissues of tomato, pepper and lettuce (Bienfait *et al.*, 2004), using an excess of $FeCl_3$ to ensure the

determination of the total amount of *o,o*-EDDHA as Fe(III)-*o,o*-EDDHA with HPLC-UV/VIS. Radioactivity assays with ^{55}Fe(III)-^{14}C-EDDHA (Tiffin *et al.*, 1961) and ^{59}Fe(III)-^{14}C-EDDHA (Römheld and Marschner, 1981) confirmed plant uptake of EDDHA and Fe(III)-EDDHA in soybean, sunflower, pea, peanut and some grasses (millet, wheat and corn). No differences in ^{14}C uptake between Fe-deficient and Fe-sufficient plants were found. However, the ^{59}Fe/^{14}C ratios were 250-fold higher in Fe-deficient vs. Fe-sufficient soybean exudates and sunflower shoots, whereas in grasses the ^{59}Fe/^{14}C ratio was always approximately 1.0, irrespective of the plant Fe status. Grasses secrete low molecular weight compounds, phytosideropores, that bind specifically Fe(III), and then take up the whole Fe(III)-phytosideropore complex using a specific transport protein. Until now, it is not clear whether the EDDHA/Fe(III)-EDDHA uptake in plants is through a passive or an active pathway.

Recent analytical technologies can help to understand the action mechanism of Fe(III)-chelates. The use of ICP/MS with collision cell allows the quantification of very low amounts of stable Fe isotopes in plant tissues (Rodríguez-Castrillón *et al., 2008*). The use of ^{54}Fe-, ^{57}Fe- and ^{58}Fe-chelates makes possible to distinguish the Fe supplied by the chelate and the naturally occurring ^{56}Fe. When cucumber plants were treated with ^{57}Fe(III)-*o,o*-EDDHA for 1 h they took up 5 µg ^{57}Fe g FW^{-1}, with 82%, 16% and 2% being allocated to roots, leaves, and stems, respectively (Rodríguez-Castrillón *et al., 2008*). In the same study, a better Fe translocation was observed for ^{57}Fe(III)-*o,o*-EDDHA than for ^{57}Fe(III)-*o,p*-EDDHA. Another technique recently applied to Fe plant research is HPLC-ESI/MS using high-resolution detectors such as time-of-flight (TOF) devices. This technique makes more reliable the determination of Fe(III)-chelates inside the plant, since both mass/charge (*m/z*) ratios and isotopic signatures are very specific parameters of each metal compound. Also, this technique provides better detection limits than HPLC-UV/VIS (e.g., 15-fold better for Fe(III)-*o,o*-EDDHA). For instance, Fe(III)-*o,o*-EDDHA was specifically determined in tomato xylem sap by using an HPLC-ESI/MS(TOF) method (Álvarez-Fernández *et al.*, 2007) using ^{57}Fe(III)-*o,o*-EDDHA as internal standard (Figure 2-3). The two stereoisomer forms of Fe(III)-*o,o*EDDHA (*racemic* and *meso*) were separated chromatographically, thus allowing monitorisation of the amounts of the two isomeric forms inside the plant. The *o,o*-EDDHA bound to two different Fe stable isotopes can also be distinguished with this technique (see insets in Figure 2-3 for the signals at *m/z* values of 412.0 and 413.0 for ^{56}Fe(III)- and ^{57}Fe(III)-*o,o*-EDDHA, respectively). This technique could also permit speciation of chelating agent (EDDHA) and metal (stable metal isotopes) forms in plant tissues.

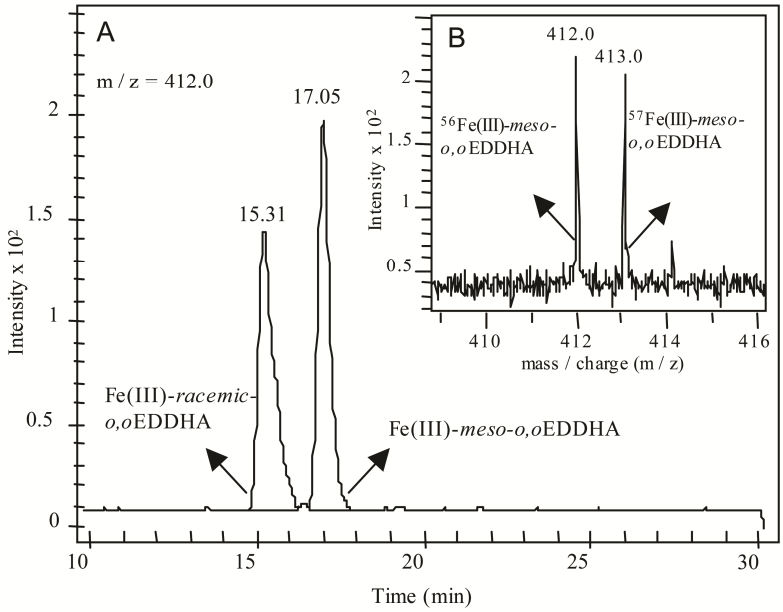

Figure 2-3. Chromatogram (Panel A) and mass spectrum (Inset, Panel B) of a xylem sap sample extracted from tomato plants treated with Fe(III)-*o,o*-EDDHA. The inset (Panel B) shows a zoomed mass spectrum (409-416 *m/z*) at a retention time of 17.05 min.

ENVIRONMENTAL IMPLICATIONS OF SYNTHETIC Fe(III)-CHELATES

The environmental implications of the use of synthetic Fe(III)-chelates have been less studied, and few studies are available on the environmental persistence, degradation and toxicity. Photodegradation studies of Fe(III)-chelates were carried out for EDTA and DTPA, whereas only one recent study deals with the photochemical and redox behaviour of Fe(III)-*o,o*-EDDHA at different pHs (Gómez-Gallego *et al.*, 2005). At typical environmental pH values (4-8), the low reduction potential of Fe(III)-*o,o*-EDDHA makes it unreactive in photochemically- or chemically-induced electron transfer processes, which invalidates photodegradation as an alternative mechanism for environmental elimination. The persistence of chelates in the soil depends on polarity and solubility (following the order: Fe(III)-EDDHSA > Fe(III)-EDDCHA >> Fe(III)-*o,o*-EDDHA > Fe(III)-*o,o*-EDDHMA), which controls the movement of the Fe(III)-chelates to lower soil layers with an excess of water (Lucena, 2003).

Phytotoxicity studies are less common and are based on the appearance of the plant toxicity symptoms (necrosis, necrotic spots, leaf malformations, etc.) and biomass decrease, as well as changes in leaf mineral composition (Fe, Mn, Zn, Cu, P, etc.). Although in plants treated with Fe(III)-chelates the concentration of Fe in leaves usually increases, a poor correlation between leaf Fe concentration and the severity of toxicity symptoms is commonly found (Broschat and Moore, 2004). Iron(III)-EDDHA toxicity frequently causes a reddish stain in the foliage. In bean plants the phytotoxicity level was 4 mM Fe(III)-EDDHA in nutrient solution (Wallace and Wallace, 1983), whereas in African marigold and zonal geranium plants showed mildly toxic effects at 1 mM Fe(III)-EDDHA concentrations, with moderately toxic effects appeared at 2 mM and 4 mM (Broschat and

Moore, 2004). This study also found that Fe-EDDHA is less toxic than Fe-EDTA and Fe-DTPA, and slightly more toxic than $FeSO_4$.

The toxicological effects of EDDHA have been mainly studied in medical applications when this chelating agent is used as a Fe chelating drug for patients with hemochromatosis. The *o*,*o*-EDDHA median lethal doses (LD_{50}) was 53 mg kg^{-1} for intervenal treated rats and mice (Rosenkrantz *et al.*, 1986) and 0.30 mg cm^{-2} of soil for slugs (*Deroceras reticulatum)* eggs exposed for 12 d (Iglesias *et al.*, 2002).

FUTURE RESEARCH

Application of the most recent analytical techniques offer an excellent tool to increase the knowledge concerning the biological and environmental implications of fertilisation with synthetic Fe(III)-chelates. A better understanding of the action mechanisms could rationalise its use, improving efficiency and minimizing environmental effects. Finally, the presence of these xenobiotic compounds in plants makes necessary to study of the toxicological effects and persistence in edible plant parts.

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Capítulo 3

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DETERMINATION OF SYNTHETIC FERRIC CHELATES USED AS FERTILIZERS BY LIQUID CHROMATOGRAPHY-ELECTROSPRAY/MASS SPECTROMETRY IN AGRICULTURAL MATRICES

Ana Álvarez-Fernández*, Irene Orera*, Javier Abadía and Anunciación Abadía

Plant Nutrition Department, Estación Experimental de Aula Dei, Consejo Superior de Investigaciones Científicas (CSIC), Apdo. 202, 50080 Zaragoza, Spain.

*Both authors contributed equally to the present work.

DETERMINATION OF SYNTHETIC FERRIC CHELATES USED AS FERTILIZERS BY LIQUID CHROMATOGRAPHY-ELECTROSPRAY/MASS SPECTROMETRY IN AGRICULTURAL MATRICES

Abstract: A high performance liquid chromatography-electrospray ionization/mass spectrometry (time of flight) method has been developed for the simultaneous determination of synthetic Fe(III)-chelates used as fertilizers. Analytes included the seven major Fe(III)-chelates used in agriculture, Fe(III)-EDTA, Fe(III)-DTPA, Fe(III)-HEDTA, Fe(III)-CDTA, Fe(III)-*o,o*EDDHA, Fe(III)-*o,p*EDDHA and Fe(III)-EDDHMA, and the method was validated using isotope labeled ^{57}Fe(III)-chelates as internal standards. Calibration curves had R values in the range 0.9962-0.9997. Limits of detection and quantification were in the ranges of 3-164 and 14-945 pmol, respectively. Analyte concentrations could be determined between the limits of quantification and 25 µM (*racemic* and *meso* Fe(III)-*o,o*EDDHA and Fe(III)-EDDHMA) or 50 µM (Fe(III)-EDTA, Fe(III)-HEDTA, Fe(III)-DTPA, Fe(III)-CDTA and Fe(III)-*o,p*EDDHA). The intraday repeatability values were approximately 0.5 and 5% for retention time and peak area, respectively, whereas the interday repeatability values were approximately 0.7 and 8% for retention time and peak area, respectively. The method was validated using four different agricultural matrices, including nutrient solution, irrigation water, soil solution and plant xylem exudates, spiked with Fe(III)-chelate standards and their stable isotope-labeled corresponding chelates. Analyte recoveries found were in the ranges of 92-101% (nutrient solution), 89-102% (irrigation water), 82-100% (soil solution) and 70-111% (plant xylem exudates). Recoveries depended on the analyte, with Fe(III)-EDTA and Fe(III)-DTPA showing the lowest recoveries (average values of 87 and 88%, respectively, for all agricultural matrices used), whereas for other analytes recoveries were between 91 and 101%. The method was also used to determine the real concentrations of Fe(III)-chelates in commercial fertilizers. Furthermore, the method is also capable to resolve two more synthetic Fe(III)-chelates, Fe(III)- EDDHSA and Fe(III)-EDDCHA, whose exact quantification is not currently possible due to the lack of commercial standards.

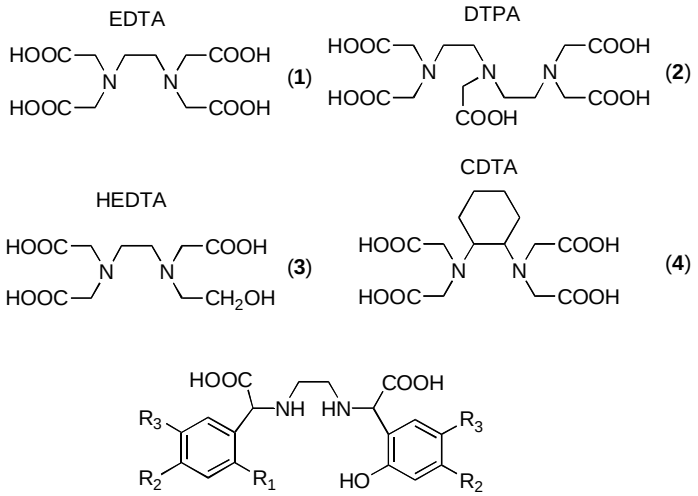
Keywords: EDDHA; EDTA; ferric chelates; fertilizers; irrigation water; liquid chromatography; mass spectrometry; nutrient solution; plant xylem; soil solution

1. INTRODUCTION

Iron deficiency is a widespread plant nutritional disorder in many areas worldwide [1-2], causing decreases in the yield and quality of crops [2-3] and being also a major problem in human nutrition [4]. The use of synthetic Fe(III)-chelates has been proven to be a successful way to provide Fe to plants since the 1950's. In spite of their high cost, fertilizers containing synthetic Fe(III)-chelates are nowadays commonly used in soil-less horticulture as well as in high value, field-grown crops affected by Fe deficiency. Synthetic Fe(III)-chelates used as fertilizers are generally derivatives from the family of ethylenediamine-carboxylic acids, and include the Fe(III)-chelates of ethylenediamine tetraacetic acid (EDTA) (**1**), di-ethylenetriamine pentaacetic acid (DTPA) (**2**), N-(2-hydroxyethyl)ethylenediaminetriacetic acid (HEDTA) (**3**), ciclohexane-1,2-diaminetetraacetic acid (CDTA) (**4**), ethylenediamine-N-N'bis(*o*-hydroxyphenylacetic) acid (*o*,*o*EDDHA) (**5**), ethylenediamine-N-(*o*-hydroxyphenylacetic)-N'-(*p*-hydroxyphenylacetic) acid (*o*,*p*EDDHA) (**6**), ethylenediamine-N-N'bis(2-hydroxy-4-methylphenylacetic) acid (EDDHMA) (**7**), ethylenediamine-N-N'bis(5-carboxy-2-hydroxyphenylacetic) acid (EDDCHA) (**8**) and ethylenediamine-N-N'bis(2-hydroxy-5-sulfophenylacetic) acid (EDDHSA) (**9**) [5]. These compounds can be applied either to the root system (via soil or nutrient solution) or to the plant shoots (via foliar spray or trunk injections). The effectiveness of these compounds is mainly based on their ability to maintain Fe in soluble forms in aerobic environments at the pH values occurring in soils and plant tissues. These chelates are stable in different pH ranges, depending on the specific formation constant of each compound and the presence of cations other than Fe(III) [5].

Aminopolycarboxylate chelating agents such as those cited above are currently under scrutiny due to their influence on metal availability and mobility, and in particular due to their high persistence in the environment [6-7]. However, the mechanisms by which plants take up Fe from these compounds and the time-span of their presence in the plant-soil-environment system are still matter of speculation. This is in part due to the lack of analytical methods able to determine in a specific, reliable and direct way the very low concentrations of synthetic Fe(III)-chelates which occur in environmental matrices as a result of Fe fertilizer applications.

Up to now, methods developed to determine simultaneously several synthetic Fe(III)-chelates have focused mainly on the analysis of simple solutions or commercial fertilizers. Various analytical techniques have been used, such as paper, gel and thin-layer chromatography, electrophoresis, gas chromatography and high performance liquid chromatography (HPLC), all of them combined to UV-Vis or atomic absorption spectroscopy [8-13]. All these methods permit reliable detection provided very good chromatographic separations are achieved, using analytical detection techniques with relatively low selectivity. These methods have focused on getting analysis times as short as possible, using pH, buffer and solvent conditions not affecting Fe-complexation during separation. However, little attention has been given until now to obtain low limits of detection and to avoid interferences in the analysis of real samples, both issues being crucial for quantifying accurately these analytes in complex matrices.



	R_1	R_2	R_3	
o,*o*EDDHA	OH	H	H	(**5**)
*o,p*EDDHA	H	OH	H	(**6**)
EDDHMA	OH	CH_3	H	(**7**)
EDDCHA	OH	H	COOH	(**8**)
EDDHSA	OH	H	HSO_3	(**9**)

Recently, more selective and sensitive analytical techniques such as inductively coupled mass spectrometry (ICP/MS) and electrospray mass spectrometry (ESI/MS) have been used, permitting to differentiate among different metal species co-eluting within a given chromatographic peak. These techniques allow for the simultaneous determination of several elements (ICP/MS) or metal-chelate molecules (ESI/MS). ICP/MS is less selective than ESI/MS, but offers higher sensitivity and a larger dynamic range [14]. A problem when using ESI/MS in the analysis of environmental matrices is the poor tolerance to non-volatile salts, which may reduce sensitivity. Both ICP/MS and ESI/MS are usually coupled to separation techniques, mainly HPLC or capilary electrophoresis, to add molecular specificity (ICP/MS and ESI/MS) and to increase detection limits in salt-rich environmental matrices (ESI/MS). Most of the methods developed so far using these techniques have generally focused on EDTA and DTPA, generally ignoring other chelates and agricultural matrices [6]. For instance, metal-EDTA, -DTPA and -CDTA complexes, including Fe(III)-EDTA, were determined in nutrient solutions and in ground and surface waters by HPLC-ICP/MS [15-16]. ESI/MS has been proven to be an useful tool in the examination of metal-EDTA complexes, including Fe(III)-EDTA [17]. Metal-EDTA complexes were also analyzed by HPLC-ESI/MS in soil solution and plant xylem samples, although Fe(III)-EDTA could not be detected because Fe from Fe(III)-EDTA precipitates as an Fe oxide at the very high (9.9) mobile phase pH used [18]. HPLC-ESI/MS was also used to determine EDTA in industrial effluents by forming the Fe(III)-EDTA complex [19], as well as to determine EDTA and DTPA in influents and effluents of wastewater treatment plants by measuring the $[M - H]^{-1}$ ions and the corresponding Fe(III) adducts [20]. The chemical characterization of fertilizers containing synthetic Fe(III)-chelates of EDTA, DTPA, EDDHA, EDDHMA, EDDHSA and EDDCHA has been tackled using HPLC-ESI/MS [21], although this study provided very limited analytical information, reporting only chromatographic retention times and *m/z* values for the $[M - H]^{-1}$ ions of each chelate. Also, HPLC-ESI/MS was used to characterize Fe(III)-EDDHSA commercial fertilizers, finding a

peak with *m/z* attributable to an Fe(III)-EDDHSA condensation product along with the peak of the active ingredient (Fe(III)-EDDHSA) [22].

The aim of this work was to develop and validate a reliable, direct and sensitive method to determine simultaneously different synthetic Fe(III)-chelates being currently used as fertilizers. The method developed is capable of analyzing the seven major Fe(III)-chelates used in agriculture, which account for a very large portion of the Fe(III)-chelate fertilizer market (247 out of the 263 products in the 2005 market in Spain). The method has been validated for its use with four agricultural matrices: nutrient solution, irrigation water, soil solution and plant xylem exudate. Furthermore, the method is also capable to resolve two more synthetic Fe(III)-chelates, Fe(III)-EDDHSA and Fe(III)-EDDCHA, whose exact quantification is not yet possible due to the lack of commercial standards.

2. EXPERIMENTAL

2.1 Chemicals and reagents

All eluents, buffers and standard solutions were prepared with analytical grade type I water (Milli-Q Synthesis, Millipore, Bedford, MA, USA). Reagent-grade glacial acetic acid, hydrochloric acid (35%), calcium carbonate and ammonium hydroxide (25%) were purchased from Panreac Química S.A (Barcelona, Spain). Ammonium acetate (99.99%, Sigma), Li hydroxide monohydrate (99.995%, Aldrich), methionine (99%, Sigma), leucine enkephalin (Tyr-Gly-Gly-Phe-Leu, 98%, Sigma), formic acid (50%, Fluka), and methanol and 2-propanol (both LC-MS grade, Riedel-de-Haën) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Glutathione (99%) was purchased from Calbiochem (San Diego, CA, USA).
Chelating agents used were $Na_2H_2EDTA \cdot 2H_2O$ (99%, Merck, Barcelona, Spain), DTPA (99%, Merck), Na_3HEDTA (99%, Merck), $CDTA \cdot H_2O$ (99%, Merck), *o,o*-EDDHA (98%, LCG Promochem, Barcelona, Spain), EDDHMA (98%, LCG Promochem) and Fe(III)-EDDCHA and Fe(III)-EDDHSA (both 5.9% w/w Fe), provided by Prof. J. M. García-Mina (Universidad de Navarra, Spain). *o,o*-EDDHA enriched in *racemic* form, *o,o*EDDHA enriched in *meso* form and *o,p*EDDHA (94.7%) were kindly provided by Prof. J.J. Lucena (Universidad Autónoma de Madrid, Spain). Labeled ^{57}Fe oxide (Fe_2O_3, 98% Fe, 95.06% ^{57}Fe) was obtained from Cambridge Isotope Laboratories (Andover, MA, USA).

2.2 Standard preparations

Solutions for tuning the mass spectrometrer were i) 10 mM LiOH, 0.2% (v/v) formic acid and 50% (v/v) 2-propanol, and ii) 1 µM leucine-enkephalin, 20 µM methionine, 5 µM glutathione, 0.1% (v/v) formic acid and 50% (v/v) methanol.

Stock solutions of ^{57}Fe-labeled (0.5 mM) and non-labeled (1.0 mM) Fe(III)-chelates were prepared by adding slowly acidic Fe solutions (36 mM Fe or 9 mM ^{57}Fe in 15% HCl; in 5% excess over the molar amount of chelating agent) over high-pH chelating agent solutions [13]. During the Fe addition, the solution pH was maintained in the range 6-8 by adding NH_4OH. Then, solutions were neutralized (to pH 7.0 with NH_4OH and HCl), equilibrated overnight in the dark and at room temperature, filtered through a 0.45 µm PVDF membrane and finally made up to volume with Milli-Q water. Stock solutions of ^{57}Fe-labeled and non-labeled Fe(III)-chelates were stored in the dark at 4 ºC. Iron(III)-

chelate standard solutions of concentrations lower than 100 µM were prepared daily from the stocks.

2.3 Agricultural matrices

In order to validate the method, recovery assays were carried out for each Fe(III)-chelate using four agricultural matrices (nutrient solution, irrigation water, soil solution and plant xylem exudate). The nutrient solution matrix was half-strength Hoagland nutrient solution [23] supplemented with 1 g l^{-1} $CaCO_3$, without Fe, pH 7.2. Irrigation water was sampled from the "Bardenas" irrigation channel, which irrigates a large agricultural area in Aragón, Northern Spain. Main irrigation water characteristics were pH 8.5, 0.35 dS m^{-1} electrical conductivity and 1.33, 2.15, 0.42, 0.03, 0.44, 2.36, 0.42, 0.61 mg l^{-1} of Ca^{2+}, Mg^{2+}, Na^{+}, K^{+}, CO_3^{2-}, HCO_3^{-}, SO_4^{2-} and Cl^{-}, respectively. A saturated-paste soil solution was obtained after water incubation of a soil sampled in a peach orchard located in Alcañiz (Teruel, Spain). Main soil characteristics were silt-sandy texture, pH in water 8.0, 30.5% total $CaCO_3$ and 0.8% organic matter. Plant xylem exudates were isolated from commercial peach trees grown in the field, following the Schölander chamber method [24]. All agricultural matrices were filtered through a 0.45 µm PVDF filter previously to their use.

2.4 Commercial fertilizers

Eight commercial fertilizers, containing at least a synthetic Fe(III)-chelate, were analyzed. The following compounds were used: product A, containing Fe(III)-EDTA and 13% soluble Fe; product B, containing Fe(III)-DTPA and 0.3% soluble Fe; product C, containing Fe(III)-HEDTA and 4.1% soluble Fe; products D, E and F, containing Fe(III)-EDDHA and 6% soluble Fe; product G, containing Fe(III)-EDDHMA and 6% soluble Fe, and product H, containing Fe(III)-EDDHSA and 6% soluble Fe. All soluble Fe contents indicated are those shown in the label and are given on a w/w basis. Fertilizer stock solutions (10 mM Fe) were prepared by dissolving the products in Milli-Q water. Solutions were filtered through a 0.45 µm PVDF membrane and stored in the dark at 4 ºC.

2.5 HPLC-ESI/MS(TOF) analysis

Analyses were carried out with a BioTOF II (Bruker Daltonics, Billerica, MA, USA) coaxial multipass time-of-flight mass spectrometer (MS(TOF)) equipped with an Apollo electrospray ionization source (ESI), and coupled to a Waters Alliance 2795 HPLC system (Waters, Mildford, MA, USA). The resolution of the MS(TOF) detector used is higher than 10,000 FWHM (full width at half-maximum height).

The BioTOF II was operated with endplate and spray tip potentials of 2.8 kV and 3.3 kV, respectively, in negative ion mode, and of 3.5 kV and 4.0 kV, respectively, in positive ion mode. Drying gas (N_2) pressure was kept at 30 psi. Nebulizer gas (N_2) pressure was kept at 30 and 60 psi in ESI/MS and LC-ESI/MS experiments, respectively. The mass axis was calibrated using Li-formate adducts in negative ion mode and a mixture of 1 µM leucine-enkephaline, 5 µM glutathione and 20 µM methionine in positive ion mode. Spectra were acquired in the mass/charge ratio (*m/z*) range of 100-800.

To optimize the MS signal, direct injection of 10 µM solutions of all Fe(III)-chelates were carried out using a syringe pump (Cole-Parmer Instrument, Vernon Hills, IL, USA) operated at 2 µl min^{-1}. Optimal parameter values after tuning included negative polarity, orifice voltage value of 120 V and drying gas temperature of 200 ºC. These parameters

were chosen to maximize all signals without compromising the detection of any of the analytes.

High-performance liquid chromatography was performed with a Waters Alliance 2795 HPLC system (Waters) equipped with on-line degasser, autosampler module and column oven. Different chromatographic conditions were tested, and those described below were the best to obtain i) the best possible MS signal for all analytes in the shorter analysis time, ii) the best possible separation between analytes having the same *m/z* and iii) no changes in Fe(III)-complexation during separation. The column used was an analytical HPLC column (Symmetry® C18, 15 cm x 2.1 mm i.d., 5 µm spherical particle size, Waters) protected by a guard column (Symmetry® C18, 10 mm x 2.1 mm i.d., 3.5 µm spherical particle size, Waters). Autosampler and column temperatures were 6 and 30 ºC, respectively. Injection volume was 50 µl and flow rate was 100 µl min^{-1}. The mobile phase was built using three solvents: A (Milli-Q water), B (methanol) and C (20 mM ammonium acetate in Milli-Q water, pH 6.0). The initial conditions of the gradient program (93% A, 2% B and 5% C) were held for 3 min, followed by a linear gradient to 40% A, 55% B and 5% C until 7 min, and an isocratic step with the latter composition until 17 min. Then, in order to return to the initial conditions, a new linear gradient to 93% A, 2% B and 5% C was run until 20 min, followed by a 10-min re-equilibration with the same mobile phase composition. The HPLC apparatus was coupled to the ESI/MS(TOF) through a 125 µm i.d. PEEK tube (Upchurch Scientific, Oak Harbor, WA, USA).

The system was controlled with the software packages BioTOF (version 2.2, Bruker Daltonics) and HyStar (version 2.3, Bruker DaltoniK, Bremen, Germany). Data were processed with Data Analysis software (version 3.2, Bruker DaltoniK).

Validation was carried out by obtaining calibration curves (in each case corrected by using the corresponding ^{57}Fe-labeled, Fe(III)-chelate as an internal standard), limits of detection (LODs, signal/noise (S/N) ratio of 3), limits of quantification (LOQs, S/N ratio of 10), intra- and interday repeatability and recoveries in different matrices using standard techniques (for a complete description, see Results).

3. RESULTS

3.1 ESI/MS(TOF) analysis

Mass spectra of non-labeled Fe(III)-chelate standard solutions were obtained under the ESI/MS conditions described in Materials and Methods (Fig. 3-1). Major peaks found correspond to the ^{56}Fe signal of the $[M - H]^{-1}$ ions at *m/z* values 344.0 for Fe(III)-EDTA (Fig. 3-1a), 445.0 for Fe(III)-DTPA (Fig. 3-1b), 330.0 for Fe(III)-HEDTA (Fig. 3-1c), 398.0 for Fe(III)-CDTA (Fig. 3-1d), 412.0 for Fe(III)-*o,o*EDDHA and Fe(III)-*o,p*EDDHA (Figs. 3-1e and 3-1f, respectively), and 440.0 for Fe(III)-EDDHMA (Fig. 3-1g). Minor peaks at *m/z* 380.0 for Fe(III)-EDTA (Fig. 3-1a), 366.0 for Fe(III)-HEDTA (Fig. 3-1c) and 448.0 *m/z* for Fe(III)-*o,p*EDDHA (Fig. 3-1f) correspond to the ^{56}Fe signal of the chloride adduct $[M + Cl]^{-1}$ ions. Also, a minor peak at 354.0 *m/z* for Fe(III)-CDTA (Fig. 3-1d) is due to the ^{56}Fe signal of the mono-decarboxylation of the analyte $[M - H - CO_2]^{-1}$.

In the positive ion mode, major peaks found in the MS spectra were at *m/z* values 346.0, 447.0, 332.0, 400.0, 414.0, 414.0 and 442.0 corresponding to the ^{56}Fe signal of the $[M + H]^{+1}$ ions (for Fe(III)-EDTA, Fe(III)-DTPA, Fe(III)-HEDTA, Fe(III)-CDTA, Fe(III)-*o,o*EDDHA, Fe(III)-*o,p*EDDHA and Fe(III)-EDDHMA, respectively, not shown). Signals

obtained in positive mode were slightly less intense (with a lower S/N) than those obtained in the negative ion mode (data not shown). Also, in the positive ion mode diluted acids (formic or acetic) had to be used to assist in the formation of positively charged gas phase ions, which may compromise the stability of the Fe(III)-chelates. Therefore, the negative ion mode was chosen for further experiments.

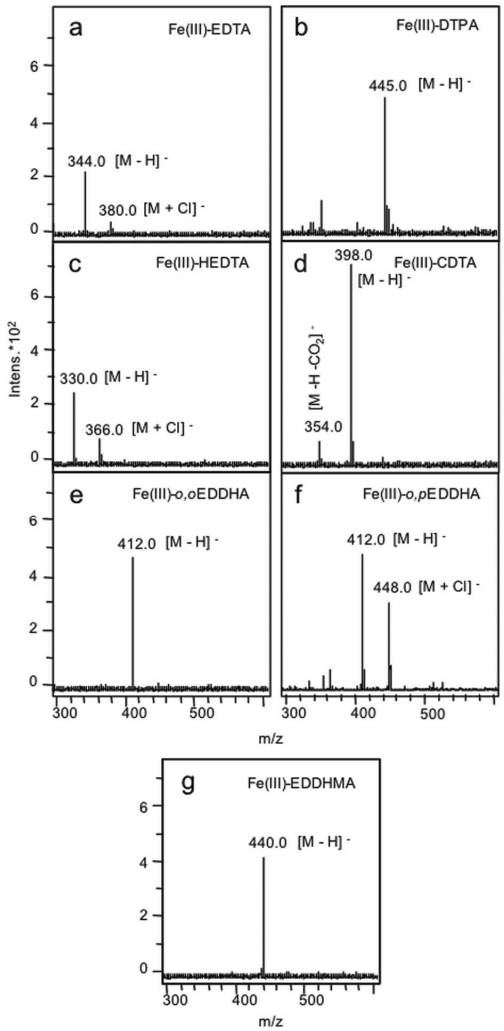


Figure 3-1. ESI/TOF mass spectra of Fe(III)-EDTA (a), Fe(III)-DTPA (b), Fe(III)-HEDTA (c), Fe(III)-CDTA (d), Fe(III)-*o,o*EDDHA (e), Fe(III)-*o,p*EDDHA (f) and Fe(III)-EDDHMA (g) in negative ion mode. Data were acquired by injecting 5 µM solutions of each analyte in water, except for Fe(III)-*o,p*EDDHA, where solution concentration was 20 µM.

3.2 HPLC-ESI/MS(TOF) analysis

Analytes were separated with a solvent gradient at pH 6.0 in a C_{18} column, and mass spectra were acquired by ESI/MS(TOF) in the *m/z* range of 100-800 during the whole chromatographic run, to obtain three-dimensional (time, *m/z*, and intensity) chromatograms. For each Fe(III)-chelate, the ion chromatogram was extracted at the *m/z* of the ^{56}Fe isotope

signal of the $[M - H]^{-1}$ molecular ion with a ±0.2 *m/z* precision range, except for Fe(III)-*o,p*EDDHA, for which both the *m/z* of the ^{56}Fe isotope signal of the $[M - H]^{-1}$ ion and that of the $[M + Cl]^{-1}$ ion were used ($[M - H]^{-1}$ and $[M + Cl]^{-1}$ were the two major main ions in the Fe(III)-*o,p*EDDHA spectra). Results show that the HPLC-ESI/MS(TOF) method developed has high selectivity, allowing to resolve adequately all Fe(III)-chelates tested (Fig. 3-2a). Retention times were 4.9 min for Fe(III)-DTPA, 5.1 min for Fe(III)-EDTA, 5.3 min for Fe(III)-HEDTA, 12.0 min for Fe(III)-CDTA, 14.6 min for *racemic* Fe(III)-*o,o*EDDHA, 15.7 min for Fe(III)-*o,p*EDDHA, 16.1 min for a first stereoisomer of Fe(III)-EDDHMA, 16.7 min for *meso* Fe(III)-*o,o*EDDHA and 17.9 min for a second stereoisomer of Fe(III)-EDDHMA. The two peaks of Fe(III)-EDDHMA are likely the *racemic* and *meso* forms, but they could not be assigned because of the lack of standards. Although they have the same *m/z*, the three Fe(III)-EDDHA compounds (Fe(III)-*o,p*EDDHA, *racemic* and *meso* Fe(III)-*o,o*EDDHA) were adequately separated by HPLC. In all cases, ^{57}Fe isotopically-labeled Fe(III)-chelates co-eluted with their corresponding unlabeled Fe(III)-chelates (Fig. 3-2b). Times for separation and column stabilization were approximately 20 and 10 min, respectively, thus leading to a total analysis run time of 30 min per sample.

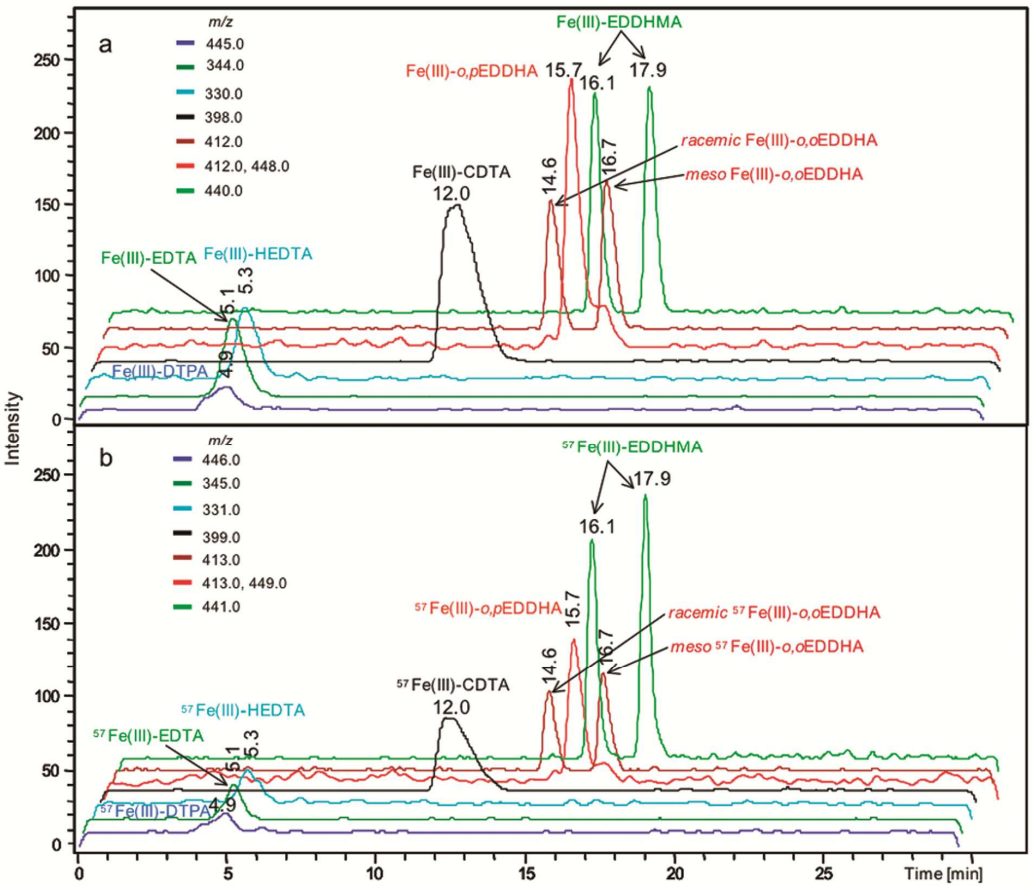


Figure 3-2. Chromatograms of simple solutions of non-labeled (a) and the corresponding ^{57}Fe-labeled (b) Fe(III)-chelates. Non-labeled Fe(III)-chelates were at concentration of 20 µM, except for Fe(III)-EDDHMA, used at concentration of 10 µM. ^{57}Fe-labeled Fe(III)-chelates were at concentration of 5 µM, except for Fe(III)-DTPA, used at concentration of 25 µM. Solutions were made in the mobile phase used at the start of the elution gradient (1 mM ammonium acetate, pH 6.0, 2% (v/v) methanol).

3.3 Validation of the HPLC-ESI/MS(TOF) method

The HPLC-ESI/MS(TOF) method was validated preparing solutions of Fe(III)-chelate standards in initial mobile phase (1 mM ammonium acetate, pH 6.0, 2% (v/v) methanol). Calibration curves corrected with internal standardization, LODs, intra- and interday repeatability, and recovery in agricultural matrices were measured.

Calibration curves corrected by internal standardization were obtained by analyzing solutions of standards in the ranges of 2-50 µM (Fe(III)-EDTA and Fe(III)-HEDTA), 5-50 µM (Fe(III)-DTPA), 0.5-50 µM (Fe(III)-CDTA and Fe(III)-*o,p*EDDHA), and 0.25 to 25 µM (*racemic* and *meso* Fe(III)-*o,o*EDDHA and Fe(III)-EDDHMA). The corresponding ^{57}Fe-labeled Fe(III)-chelates were used as internal standards. The peak area at the *m/z* corresponding to the $[M - H]^{-1}$ the ^{57}Fe-chelate also include a small contribution of the non-labeled Fe(III)-chelate, because the natural isotopic composition of the analyte. To calculate the peak area ratios (sample area/area of the internal standard) used in the calibration curves, the natural contribution of the non-labeled analyte at the *m/z* $[M - H]^{-1}$ of the ^{57}Fe-labeled internal standard was subtracted from the total peak area. In all cases, data were fitted to a linear regression (R of 0.9962-0.9997) (Fig. 3-3) indicating that the analytes could be determined in those ranges of concentrations.

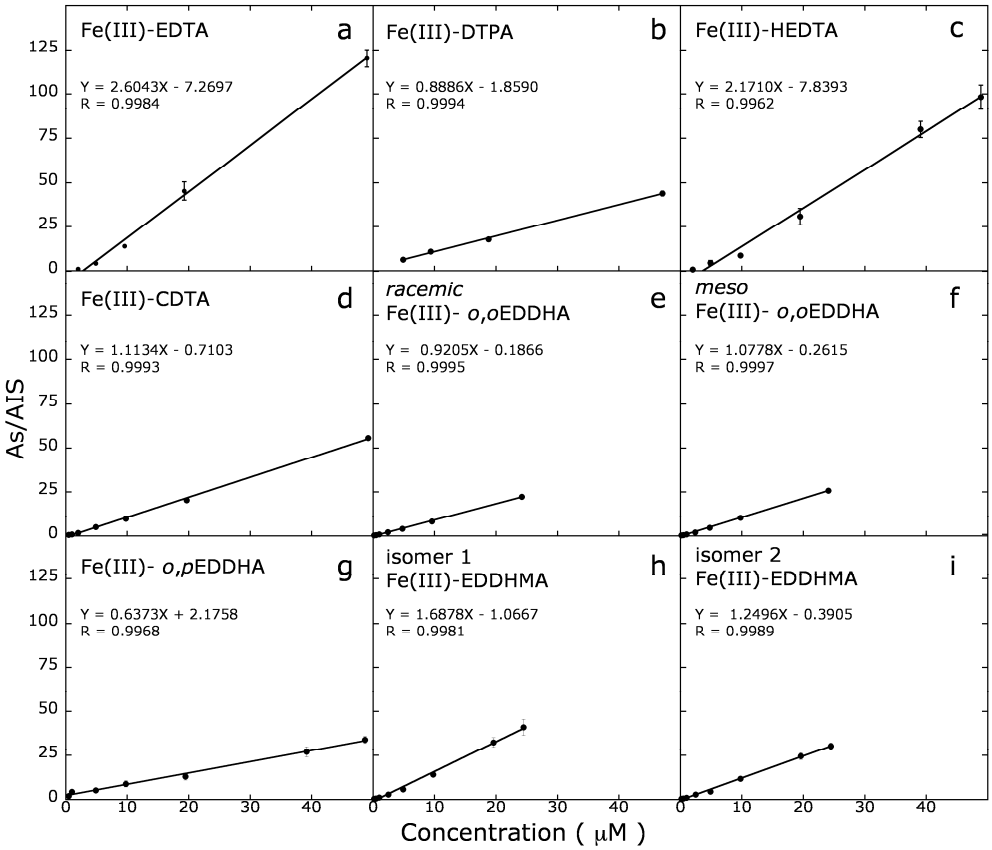


Figure 3-3. Calibration curves of Fe(III)-EDTA (a), Fe(III)-DTPA (b), Fe(III)-HEDTA (c), Fe(III)-CDTA (d), *racemic* Fe(III)-*o,o*EDDHA (e), *meso* Fe(III)-*o,o*EDDHA (f), Fe(III)-*o,p*EDDHA (g), isomer 1 of Fe(III)-EDDHMA (h) and isomer 2 of Fe(III)-EDDHMA (i) obtained by plotting the peak area ratio (sample area/area of the internal standard; As/AIS; Y-axis) vs. the Fe(III)-chelate concentration injected. Internal

standards were at a concentration of 5 µM, except for ^{57}Fe(III)-DTPA, used at a concentration of 25 µM. Solutions were made in the mobile phase used at the start of the elution gradient (1 mM ammonium acetate, pH 6.0, 2% (v/v) methanol). Bars are SE for triplicate measurements.

LODs, defined as the analyte amounts giving an S/N ratio of 3, were between 3 to 164 pmol, the lowest value corresponding to the second isomer of Fe(III)-EDDHMA and the highest to Fe(III)-DTPA (Table 3-1). Using a 50-µl injection volume, these values are equivalent to analyte concentrations (in the injected solution) in the range of 0.1-3.3 µM. LOQs, defined as the amounts giving an S/N ratio of 10, ranged from the lowest value of 14 pmol for the second isomer of Fe(III)-EDDHMA to the highest value of 945 pmol for Fe(III)-DTPA (Table 3-1).

Table 3-1. Limits of detection (LOD) and quantification (LOQ) for several synthetic Fe(III)-chelates used as fertilizers.

Analyte	LOD (pmol)[a]	LOQ (pmol)[b]
Fe(III)-EDTA	123	328
Fe(III)-DTPA	164	945
Fe(III)-HEDTA	87	295
Fe(III)-CDTA	10	50
Fe(III)- *o,o*EDDHA		
racemic	4	19
meso	4	19
Fe(III)-*o,p*EDDHA	4	17
Fe(III)-EDDHMA		
Isomer 1	5	17
Isomer 2	3	14

[a]LOD, defined as the analyte amount giving a signal/noise (S/N) ratio of 3.
[b]LOQ, defined as the analyte amount giving an S/N ratio of 10.

The intraday repeatability of the HPLC-ESI/MS(TOF) method was assessed from 6 consecutive chromatographic runs, using two levels of concentration for each analyte: 10 and 50 µM for Fe(III)-EDTA, Fe(III)-HEDTA and Fe(III)-*o,p*EDDHA, 20 and 75 µM for Fe(III)-DTPA, 2 and 20 µM Fe(III)-CDTA, and 1 and 10 µM for *meso* and *racemic* Fe(III)-*o,o*EDDHA and Fe(III)-EDDHMA. The variation in retention time and peak area ratio was assessed for each analyte (Table 3-2). The interday repeatability of the method was also assessed, by analyzing the same standard solution for six consecutive days (Table 3-2).

Table 3-2. Intra- (n = 6) and interday (n = 6) repeatability (RSD %) of the HPLC-ESI/MS(TOF) method.

Analyte	Concentration (µM)	Intraday		Interday	
		R.T. (min)	As/AIS	R.T. (min)	As/AIS
Fe(III)-EDTA	10	0.1	8.6	1.4	9.8
	50	0.9	5.0	1.1	5.3
Fe(III)-DTPA	20	1.3	5.6	1.2	6.8
	75	1.1	2.5	0.8	4.1
Fe(III)-HEDTA	10	0.9	3.0	0.9	5.7
	50	0.8	2.4	1.3	4.3
Fe(III)-CDTA	2	0.6	6.2	0.6	10.6
	20	0.4	4.9	0.8	8.3
Fe(III)-*o,o*EDDHA					
racemic	1	0.6	6.6	0.4	10.2
	10	0.9	5.9	0.6	9.8
meso	1	0.1	7.4	0.3	9.4
	10	0.3	4.8	0.4	8.3
Fe(III)-*o,p*EDDHA	10	0.3	8.2	1.3	8.9
	50	0.1	7.9	1.0	10.2
Fe(III)-EDDHMA					
Isomer 1	1	0.3	4.4	0.2	6.2
	10	0.5	5.2	0.3	7.4
Isomer 2	1	0.0	3.6	0.3	6.0
	10	0.3	5.5	0.4	6.3

RSD, relative standard deviation; R.T., retention time; As/AIS, peak area ratios (sample area/area of the internal standard).

Solutions were made in mobile phase at the initial conditions of the elution gradient (1 mM ammonium acetate, pH 6.0, 2% (v/v) methanol) and contained 5 µM of the corresponding ^{57}Fe-labeled Fe(III)-chelate as an internal standard (except for Fe(III)-DTPA solution, which contained 25 µM of ^{57}Fe(III)-DTPA).

The relative standard deviation (RSD) for peak retention time always was lower than 1.3% in the intraday test and 1.4% in the interday test. The RSD for peak area ratio was in the range of 2.4-8.6% in the intraday test and 4.1-10.6% in the case of the interday test.

Recovery assays were carried out for each Fe(III)-chelate by spiking four different agricultural matrices (nutrient solution, irrigation water, soil solution and plant xylem exudate) with known amounts of each non-labeled Fe(III)-chelate, using in each case the corresponding ^{57}Fe-labeled Fe(III)-chelate as an internal standard. Representative chromatograms for the analysis of Fe(III)-*o,o*EDDHA in agricultural matrices are shown in Fig. 3-4.

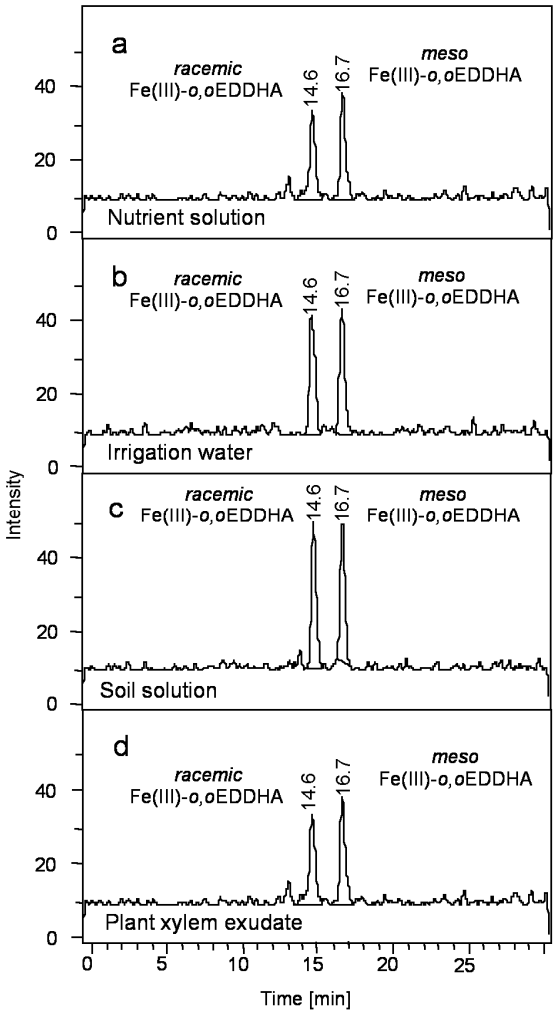


Figure 3-4. Chromatograms at 412.0 *m/z* of nutrient solution (a), irrigation water (b), soil solution (c) and plant xylem exudate (d) spiked with 10 µM Fe(III)-*o,o*EDDHA.

All Fe(III)-chelates had similar retention times in agricultural matrices than in simple solutions. Analyte recoveries found were in the ranges of 92-101% for nutrient solution, 89-102% for irrigation water, 82-100% for soil solution and 70-111% for plant xylem exudate, respectively (Table 3-3). Recoveries depended on the analyte, with Fe(III)-EDTA and Fe(III)-DTPA showing the lowest recoveries (average values of 87 and 88%, respectively, for all agricultural matrices used) and on the agricultural matrices tested, with the lowest recoveries found for soil solution and plant xylem exudate, with average recovery values of 90 and 91%, respectively (average).

Table 3-3. Recoveries (in %) obtained for the 9 different Fe(III)-chelates using different agricultural matrices.

Analyte	Nutrient solution	Irrigation water	Soil solution	Plant xylem exudate
Fe(III)-EDTA	92.1±2.0	88.9±2.5	82.6±2.3	82.8±1.8
Fe(III)-DTPA	101.2±4.0	100.2±5.7	81.9±4.8	70.0±5.0
Fe(III)-HEDTA	98.9±2.8	101.7±3.0	83.3±9.8	96.9±0.9
Fe(III)-CDTA	99.5±5.4	94.0±5.0	98.6±2.9	110.9±9.9
[a]Fe(III)-*o,o*EDDHA	(94.8±3.0	(96.6±1.6)	(91.6±1.1)	(95.6±1.1)
racemic	94.4±1.5	99.5±2.3	104.3±2.6	95.7±4.4
meso	94.6±6.2	93.6±4.9	78.8±1.6	95.4±2.2
Fe(III)-*o,p*EDDHA	100.5±1.3	95.3±4.6	91.9±2.0	91.5±1.7
[a]Fe(III)-EDDHMA	(96.6±2.1	(93.5±2.3)	(99.9±5.4)	(89.9±2.0)
Isomer 1	94.7±2.5	97.6±3.6	103.6±5.6	86.9±1.5
Isomer 2	96.5±2.8	89.9±1.3	95.9±5.2	92.5±2.6

Values are means ± SE (n = 3).
The amounts spiked were 10 µM of Fe(III)-EDTA, Fe(III)-CDTA, Fe(III)-*o,o*EDDHA and Fe(III)-EDDHMA, and 50 µM of Fe(III)-DTPA, Fe(III)-HEDTA and Fe(III)-*o,p*EDDHA.
[a]Values for Fe(III)-*o,o*EDDHA and Fe(III)-EDDHMA are presented for the *racemic* mixture and *meso* forms, and also for the average compound (values in parenthesis), assuming a 50% content of each form.

3.4 Analysis of fertilizers

Chromatograms of commercial fertilizers containing two of the most common Fe(III)-chelates, Fe(III)-EDTA and Fe(III)-EDDHA, are presented in Fig. 3-5a and b, respectively. The analysis of a Fe(III)-EDTA commercial fertilizer showed a peak with *m/z* 344.0 at 5.1 min, corresponding to the ^{56}Fe signal of the $[M - H]^{-1}$ ion of this chelate (Fig. 3-5a). The chromatogram of a Fe(III)-EDDHA commercial fertilizer showed three peaks, all of them with *m/z* 412.0, corresponding to ^{56}Fe signal of the $[M - H]^{-1}$ ion of *racemic* and *meso* Fe(III)-*o,o*EDDHA and Fe(III)-*o,p*EDDHA, at retention times of 14.6, 16.7 and 15.7 min (Fig. 3-5b). A zoomed mass spectra at the retention time of the meso Fe(III)-*o,o*EDDHA is presented in the inset of Fig. 3-5b, as an example of how the MS technique used can resolve the peaks for the different Fe isotopes (^{54}Fe-, ^{56}Fe-, ^{57}Fe-*o,o*EDDHA) corresponding to the $[M - H]^{1-}$ ions.

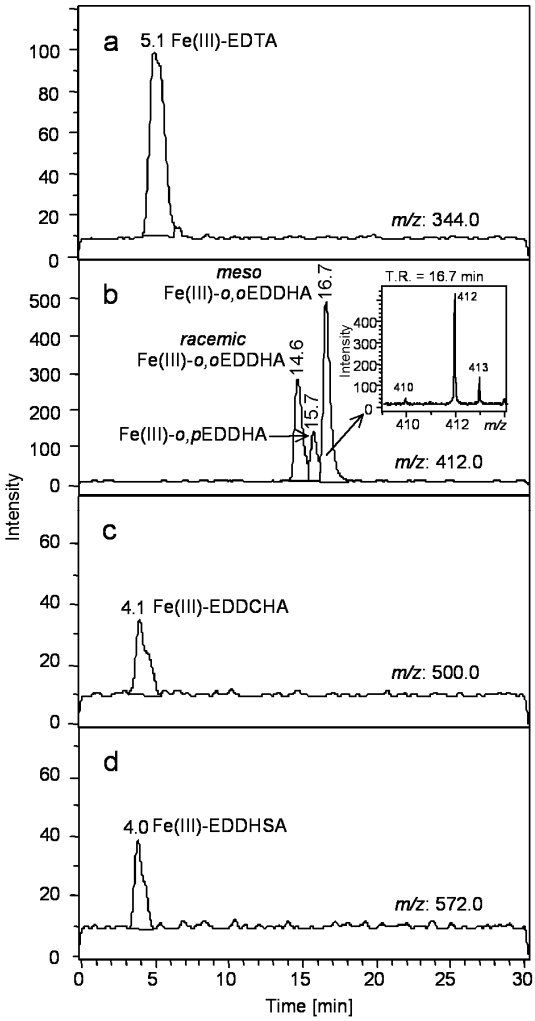


Figure 3-5. Chromatograms of commercial fertilizers. Fe(III)-EDTA (a), Fe(III)-EDDHA (b), Fe(III)-EDDCHA (c) and Fe(III)-EDDHSA (d) fertilizer solutions were at a concentration of 4.3, 10.6, 18.3 and 90.3 mg product l^{-1}, respectively, in the mobile phase used at the start of the elution gradient (1 mM ammonium acetate, pH 6.0, 2% (v/v) methanol). The inset in Fig. 3-5.b shows a zoom of the mass spectrum at a retention time of 16.7 min for the Fe(III)-EDDHA fertilizer analysis.

The amounts of Fe(III)-chelates found in the commercial fertilizers were in the range of 0.3-10.5% (w/w) (Table 3-4). These values account for 81, 107, 47, 64, 88, 62 and 57% of the soluble Fe contents declared in the label for products A, B, C, D, E, F and G, respectively. For fertilizers containing Fe(III)-EDDHA or Fe(III)-EDDHMA, the chelated Fe contents found compare well with data obtained using the European Community Official Method of analysis [25] in a study analyzing 110 Fe(III)-EDDHA and 5 Fe(III)-EDDHMA fertilizers (all of them declaring a 6% soluble Fe content, commercialized in Spain in the years 2003 and 2004). The mean for chelated Fe content of the three Fe(III)-EDDHA products analyzed in this study (3.5%) is slightly lower than the mean (4.0%) obtained using the official method [26]. The chelated Fe content value (3.4%) obtained using the HPLC-ESI/MS(TOF) method in the only Fe(III)-EDDHMA fertilizer analyzed is somewhat lower than the mean value (4%) obtained using the official method [26].

Commercial fertilizers containing Fe(III)-EDDCHA and Fe(III)-EDDHSA were also analyzed. These fertilizers showed peaks at 4.1 min (*m/z* 500.0; Fe(III)-EDDCHA) and 4.0 min (*m/z* 572.0; Fe(III)-EDDHSA), both of them attributable to the corresponding ^{56}Fe signal of the $[M - H]^{-1}$ ions (Fig. 3-5c-d). Since commercial standards of EDDHSA and EDDCHA are not commercially available, accurate quantification of these compounds cannot be carried out yet.

Table 3-4. Contents of Fe(III)-chelates found in commercial fertilizers products using the HPLC-ESI/MS(TOF) method.

Product	Fe(III)-chelate	Content (g chelated Fe / 100 g product)
A	Fe(III)-EDTA	10.50±0.65
B	Fe(III)-DTPA	0.32±0.01
C	Fe(III)-HEDTA	1.91±0.39
D		
	Fe(III)-*o,o*EDDHA	2.94±0.15
	Fe(III)-*o,p*EDDHA	0.91±0.05
E		
	Fe(III)-*o,o*EDDHA	4.32±0.21
	Fe(III)-*o,p*EDDHA	0.94±0.07
F		
	Fe(III)-*o,o*EDDHA	3.13±0.14
	Fe(III)-*o,p*EDDHA	0.58±0.08
G	Fe(III)-EDDHMA	3.41±0.11

Values are means ± SE (n = 3).

4. DISCUSSION

Synthetic Fe(III)-chelates are extensively used as Fe fertilizers, both in high-value crops grown in the field and in soil-less horticulture, making thus necessary to have reliable methods to analyze these xenobiotic compounds in agricultural matrices. In this work we have developed and validated an HPLC-ESI/MS(TOF) method capable to measure the seven major synthetic Fe(III)-chelates used as fertilizers, including Fe(III)-EDTA, Fe(III)-DTPA, Fe(III)-HEDTA, Fe(III)-CDTA, Fe(III)-*o,o*EDDHA, Fe(III)-*o,p*EDDHA and Fe(III)-EDDHMA, in several agricultural matrices. The method involves separation by reverse phase HPLC, ionization by ESI and highly selective detection of the analytes, using exact mass measurements with a TOF mass spectrometer.

This is the first time, to our knowledge, that Fe(III)-EDTA, Fe(III)-DTPA, Fe(III)-HEDTA, Fe(III)-CDTA, *racemic* Fe(III)-*o,o*EDDHA, *meso* Fe(III)-*o,o*EDDHA, Fe(III)-*o,p*EDDHA, and the two Fe(III)-EDDHMA stereoisomers are determined simultaneously and directly. The method represents significant advantages to traditional methods for the determination of synthetic Fe(III)-chelates. First, the identification of analytes is unequivocal, based on its retention time, exact *m/z* ratio and Fe isotopic signature. Also, all compounds are measured directly and simultaneously under chromatographic conditions

preserving Fe(III)-complexation occurring in the environmental matrices used, allowing for the determination of these compounds in complex mixtures and in a single run. All these features, along with the reasonably short (30 min) analysis time required per sample and the fact that the determination can be carried out in different agricultural matrices (nutrient solution, irrigation water, soil solution and plant xylem exudate) allow for the analysis of µM concentrations of synthetic Fe(III) fertilizers in the plant-soil-environment system.

The method developed has been validated for each analyte with respect to LODs, LOQs, calibration curves, reproducibility and analyte recoveries, always using isotopically labeled standards. Overall sensitivity was good, with LODs between 3 and 164 pmol (corresponding to concentrations in the injected sample of 0.1-3.3 µM), a range much better than those found with other methods aimed to determine simultaneously several synthetic Fe(III)-chelates. For instance, an ion-pair HPLC method developed to determine five of the Fe(III)-chelates studied here had LODs of 1790 pmol for Fe(III)-*o,o*EDDHA and Fe(III)-EDDHMA (for the other three analytes, Fe(III)-EDTA, Fe(III)-DTPA and Fe(III)-HEDTA, LODs were not reported) [11]. A second ion-pair HPLC method aimed to determine five synthetic Fe(III)-chelates was only capable to determine analyte concentrations above 8.9 µM [13]. On the other hand, methods have been developed to determine individual Fe(III)-chelates (often along with other analytes), and some of these had low LOD values, particularly for Fe(III)-EDTA. For instance, low LOD values (0.02 µM) were obtained in a method designed to measure EDTA as Fe(III)-EDTA by HPLC-ESI/MS [19]; this value is lower than the 2.45 µM Fe(III)-EDTA LOD obtained with our method. Very low LOD values for Fe(III)-EDTA (125-150 nM) were also obtained with a HPLC-ICP/MS method developed to determine various polycarboxylic chelators (including EDTA, CDTA and DTPA and others) and their metal complexes, although LODs for Fe(III)-CDTA and Fe(III)-DTPA were not studied [15].

The most common Fe fertilizer used in fruit crops grown in calcareous soils, Fe(III)-*o,o*EDDHA, has been much less studied than Fe(III)-EDTA. For this compound, the LOD of our method (0.08 µM) is better than the values found until now using HPLC and UV-Vis spectroscopy (1.2 µM in simple solutions and 60 µM in soil solutions [27] and 263 µM in plant tissue extracts [28]). Also, for Fe(III)-*o,p*EDDHA, a little-studied compound whose use as fertilizer has been recently accepted by the new European Community fertilizer regulation [29], the LOD obtained here (0.07 µM) is lower than the 3.3 µM LOD of the only (HPLC-Vis) method published until now [30].

The method repeatability for peak area, with RSD values of approximately 5 and 8% for intra- and interday experiments, compares well with HPLC-ESI/MS or HPLC-ICP/MS methods, although values are not as good as those obtained with methods using HPLC coupled to UV-Vis spectroscopy. For instance, the values of Fe(III)-EDTA repeatability, in the range of 5-10%, are in line with values of 5-6% found with HPLC-ICP/MS [15] and 2% obtained using HPLC-ESI/MS [19]. Methods using HPLC coupled to UV-Vis spectroscopy, however, had repeatability values of approximately 1% (Fe(III)-EDTA and Fe(III)-DTPA [9]; Fe(III)-*o,o*EDDHA [27]; Fe(III)-*o,p*EDDHA [30]), values lower than those found here for the same compounds, which are in the range of 3-10%. The recoveries obtained by spiking agricultural matrices were good, and only the recovery for Fe(III)-EDTA in all agricultural matrices tested was relatively low, in the range of 83-92%, as compared with the 96% obtained for Fe(III)-EDTA in industrial effluents [19]. Recoveries for Fe(III)-*o,o*EDDHA were in the range of 79-104%, similar to the 84-94% found by Bienfait *et al.* [28].

The method has wide possibilities of application, and it has been tested so far with different agricultural matrices (nutrient solution, irrigation water, soil solution and plant xylem exudate) and with fertilizers, showing its suitability to perform analyses in a variety of studies. Chelated Fe contents obtained for fertilizers compares well with data obtained by using the European Community Official method of analysis by García-Marco [26]. In addition to Fe(III)-EDTA, Fe(III)-DTPA, Fe(III)-HEDTA, Fe(III)-CDTA, *racemic* Fe(III)-*o,o*EDDHA, *meso* Fe(III)-*o,o*EDDHA, Fe(III)-*o,p*EDDHA, *racemic* Fe(III)-EDDHMA and *meso* Fe(III)-EDDHMA, the chelates Fe(III)-EDDHSA and Fe(III)-EDDCHA (putatively assigned to the peaks at 4.0 min with a 572.0 *m/z* and 4.1 min with a 500.0 *m/z*) could also be analyzed, therefore providing a tool for a comprehensive study of the fate, action mechanisms and possible environmental side-effects of synthetic Fe(III)-chelate fertilizers. Furthermore, the method also seems to be suitable to analyze synthetic chelates of metals other than Fe (results not shown).

In summary, the method developed permits the direct and simultaneous analysis of the major synthetic Fe(III)-chelates used as fertilizers with extreme selectivity, high sensitivity and sufficient reproducibility. The rapidity of the analysis allows for a high analysis throughput. Furthermore, the resolution of the mass spectrometer used can give information on isotopic distribution (see inset in Fig. 3-5b), allowing its use as a tool in metabolic studies with stable isotopes. For instance, using synthetic Fe(III)-chelates labeled with low-abundance Fe stable isotopes (^{54}Fe, ^{57}Fe and ^{58}Fe), the uptake pathways of these compounds applied to different parts of the plant at the same time (e.g. foliar, trunk, soil applied) can be followed. Also, the uptake rates of different synthetic Fe(III)-chelates can be studied.

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Capítulo 4

RAPID COMMUNICATIONS IN MASS SPECTROMETRY

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DETERMINATION OF *o,o*EDDHA -A XENOBIOTIC CHELATING AGENT USED IN Fe-FERTILIZERS- IN PLANT TISSUES BY LIQUID CHROMATOGRAPHY-ELECTROSPRAY MASS SPECTROMETRY: OVERCOMING MATRIX EFFECTS

Irene Orera, Anunciación Abadía, Javier Abadía and Ana Álvarez-Fernández

Plant Nutrition Department, Estación Experimental de Aula Dei, Consejo Superior de Investigaciones Científicas (CSIC), Apdo. 202, 50080 Zaragoza, Spain.

DETERMINATION OF *o,o*EDDHA -A XENOBIOTIC CHELATING AGENT USED IN Fe-FERTILIZERS- IN PLANT TISSUES BY LIQUID CHROMATOGRAPHY-ELECTROSPRAY MASS SPECTROMETRY: OVERCOMING MATRIX EFFECTS.

Abstract: The Fe(III)-chelate of ethylenediamine-N-N'bis(*o*-hydroxyphenylacetic) acid (*o,o*EDDHA) is generally considered as the most efficient and widespread Fe fertilizer for fruit crops and intensive horticulture. The determination of the xenobiotic chelating agent *o,o*EDDHA inside the plant is a key issue in the study of this fertilizer. Both the low concentrations of *o,o*EDDHA expected and the complexity of plant matrices have been important drawbacks in the development of analytical methods for the determination of *o,o*EDDHA in plant tissues. The determination of *o,o*EDDHA in plant materials has been tackled in this study by liquid chromatography coupled to mass spectrometry using several plant species and tissues. Two types of internal standards have been tested: Fe stable isotope-labeled compounds and a structural analogue compound, the Fe(III)-chelate of ethylenediamine-N-N'bis(2-hydroxy-4-methylphenylacetic) acid (*o,o*EDDHMA). Iron stable isotope-labeled internal standards did not appear to be suitable because of the occurrence of isobaric endogenous compounds and/or isotope exchange reactions between plant native Fe pools and the Fe stable isotope of the internal standard. However, the structural analogue Fe(III)-*o,o*EDDHMA is an adequate internal standard for the determination of both isomers of *o,o*EDDHA (*racemic* and *meso*) in plant tissues. The method was highly sensitive, with limits of detection and quantification in the range of 3-49 and 11-162 pmol g^{-1} fresh weight, respectively, and analyte recoveries were in the range of 74 to 116%. Using this methodology, both *o,o*EDDHA isomers were found in all tissues of sugar beet and tomato plants treated with 90 µM Fe(III)-*o,o*EDDHA for 24 hours, including leaves, roots and xylem sap. This methodology constitutes a useful tool for studies on *o,o*EDDHA plant uptake, transport and allocation.

Keywords: fertilizers, iron chelates, plants, mass spectrometry and internal standard

1. INTRODUCTION

Fertilizers containing Fe(III)-chelate derivatives from synthetic aminopolycarboxylate strong binding chelating agents have been used to alleviate Fe-deficiency problems in fruit crops and intensive horticultural systems since the 1950s.[1] Specifically, the synthetic Fe(III)-chelate of ethylenediamine-N,N'-bis(*o*-hydroxyphenylacetic) acid, commonly known as Fe(III)-*o,o*EDDHA, is considered by many authors as thecmost efficient Fe(III)-chelate to control Fe chlorosis in cropscgrown in calcareous soils.[2] The presence of *o,o*EDDHA incplants was first proposed from ^{14}C measurements in plantctissues treated with ^{14}C-labeled Fe(III)-*o,o*EDDHA, including leaves, roots, stems and xylem sap exudate from soybean,[3,4] bean,[4] pea, peanut, sunflower, millet, wheat and corn.[5] In spite of the wide use and high efficiency of these Fe(III)-chelates, the mechanisms of plant uptake, transport and allocation are not yet completely elucidated.[6] This is in part due to the lack of analytical methodologies capable of determining, in a specific way, the very low concentrations of Fe(III)-chelate occurring in complex matrices such as plant extracts.

Few attempts have been made until now to determine directly *o,o*EDDHA in plant tissue extracts. Extraction and determination of Fe(III)-*o,o*EDDHA has been reported only three times, carrying out quantification always by spectrophotometric detection in the visible (VIS) spectral range. First, Fe(III)-*o,o*EDDHA was extracted from leaves and stems of tomato with a mixture of water and amyl alcohol, and quantified directly in the plant extract by spectrophotometric detection at 480 nm.[7] Using tobacco leaves, the same extraction procedure for Fe(III)-*o,o*EDDHA was modified by the addition of Pb acetate to reduce interfering components from plant tissues.[8] More recently, *o,o*EDDHA was extracted with water from tomato, pepper and lettuce leaves and roots, and tomato and pepper fruits. In that study, excess $FeNO_3$ was added to the extracts, pH was adjusted to neutrality to reconstitute the Fe(III)-*o,o*EDDHA chelate, and two Fe(III)-*o,o*EDDHA forms, the *d,l-racemic* mixture and the *meso* isomer, were separated by high-performance liquid chromatography (HPLC) and determined by spectrophotometric detection at 480 nm.[9] The sensitivity of these methodologies was either quite poor (2.1 nmol g^{-1} of fresh weight -FW-)[9] or not determined at all.[7,8] It is important to remark that sensitivity and selectivity are important issues when determining xenobiotic compounds such as *o,o*EDDHA, because of the low concentrations expected and the complexity of the matrices analyzed.

Nowadays, reliable and sensitive detection of the Fe(III)-chelates of *o,o*EDDHA and other aminopolycarboxilate strong binding chelators could be accomplished, even in complex matrices, by using HPLC separation coupled to mass spectrometry (MS) detection (HPLC/MS).[10,11] These hyphenated techniques provide high selectivity, since they permit to differentiate among analytes and co-eluting interfering compounds within a given chromatographic peak, by using exact molecular mass determination, isotopic distribution and/or fragmentation patterns.[12] For example, *o,o*EDDHA was determined as Fe(III)-*o,o*EDDHA by HPLC/MS in commercial fertilizers[13,14] and liquid agricultural matrices such as irrigation water, soil solution, nutrient solution and peach tree xylem sap.[14] In the case of the synthetic aminopolycarboxylate ligand ethylenediamine tetraacetic acid (EDTA), Ni(II)-EDTA was determined by HPLC/MS in different plant materials such as roots, xylem sap, shoots and protoplasts of a metal hyperaccumulator plant[15] and Mn(II)-, Ni(II)-, Zn(II)- and Cd(II)-EDTA complexes were determined in xylem sap of barley.[16] HPLC/MS has been also used to determine natural metal chelates such those of nicotianamine (NA). For instance Ni(II)-NA was determined directly in xylem sap of Arabidopsis.[17]

The high selectivity of current MS detectors enables to simplify extraction procedures and chromatographic separation in HPLC/MS methods. However, the high variability in the ionization process could affect reproducibility, accuracy and sensitivity of the analysis. Changes in ionization efficiency during day-to day operation are generally controlled by using internal standards (IS), compounds structurally related or similar to the analyte. Stable isotope labelled (SIL) compounds are generally chosen as IS because of the nearly identical chemical and physical properties compared to those of the target analyte.[18] Matrix effects are considered responsible for the high variability in the ionization process, especially in electrospray (ESI)-MS analysis. A matrix effect could be defined as any change (suppression or enhancement) in ionization efficiency caused by the presence of co-eluting substances.[19] For instance, ion suppression would reduce the ion intensity of the analytes, thus affecting sensitivity.

Even when IS are used, co-eluting matrix components may cause different effects on the degree of ionization for analyte and IS, therefore affecting reproducibility and accuracy. Recent studies indicate that the use of IS, even if they are SIL analogues, does not always ensure a constant analyte/IS response ratio.[20-24] This was found in studies using D-labelled compounds as IS, and attributed to the presence of different co-eluting intereferences[22,23] and different degrees of ionization supression[24] as well as to the occurrence of isotope exchange reactions.[25] In these studies, ^{13}C-, ^{15}N- or ^{17}O-labelled analogues were considered as better options than D-labeled compounds. Unfortunately, this type of SIL is not always available, due to the necessary labor and high cost associated with chemical synthesis.

The aim of this work was to develop and validate a new procedure for the extraction and HPLC/ESI-MS time-of-flight (TOF) determination of *o,o*EDDHA in plant tissues, based on the methodology[14] recently developed and validated for Fe(III)-*o,o*EDDHA in liquid agricultural matrices. Matrix effects have been assessed using as IS both SIL analogues such as ^{54}Fe(III)-*o,o*EDDHA and ^{57}Fe(III)-*o,o*EDDHA and also a compound structurally similar to the analyte, Fe(III)-*o,o*EDDHMA. The design and validation of such methodologies will contribute to provide new insights on the traceability of *o,o*EDDHA in plant tissues.

2. EXPERIMENTAL

2.1 Chemicals and reagents

All eluents, buffers, and standard solutions were prepared with analytical grade type I water (Milli-Q Synthesis, Millipore, Bedford, MA, USA). Reagent-grade glacial acetic acid, hydrochloric acid (35%) and ammonium hydroxide (25%) were purchased from Panreac Química S.A (Barcelona, Spain). Ammonium acetate (99.99%, Sigma), Li hydroxide monohydrate (99.995%, Aldrich), formic acid (50%, Fluka), methanol and 2-propanol (both LC-MS grade, Riedel-de-Haën) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

The chelating agents used were *o,o*EDDHA (98%, LCG Promochem, Barcelona, Spain) and ethylenediamine-N-N'bis(2-hydroxy-4-methylphenylacetic) acid (*o,o*EDDHMA) (98%, LCG Promochem). Natural Fe(III), (thereafter called ^{nat}Fe, with isotopic abundances of 5.8% ^{54}Fe, 91.7% ^{56}Fe, 2.1% ^{57}Fe and 0.2% ^{58}Fe), Mn(II), Cu(II) and Zn(II) were Titrisol® metal standards (1 g of metal in 15% HCl, Merck, Darmstadt, Germany). Nickel(II) was obtained as $NiSO_4$ (Panreac), and Co(II), Mg(II) and Ca(II) were obtained as

$CoCl_2$, $MgCl_2$ and $CaCl_2$ from Sigma-Aldrich. Labelled ^{54}Fe (Fe_2O_3, 99.8% ^{54}Fe) and ^{57}Fe oxides (Fe_2O_3, 95.06% ^{57}Fe) were purchased from Cambridge Isotope Laboratories (Andover, MA, USA).

2.2 Standard solutions

One mM stock solutions of *o,o*EDDHA complexes with $^{nat}Fe(III)$, $^{54}Fe(III)$, $^{57}Fe(III)$, Ca(II), Mg(II), Cu(II), Mn(II), Ni(II), Co(II), Zn(II), and Fe(III)-EDDHMA were prepared by adding slowly inorganic solutions (in mM, 36 ^{nat}Fe, 9 ^{54}Fe, 8 ^{57}Fe, 78 Cu, 91 Mn, 76 Zn, 47 Ca, 50 Mg, 49 Ni and 50 Co in 15-30% HCl or water, in 5% excess over the molar amount of chelating agent) over high-pH solutions of chelating agents.[26] During the addition of the inorganic salt the solution pH was maintained in the range 5–8 by adding simultaneously NH_4OH. Then, solutions were adjusted to pH 7.0 with HCl, equilibrated overnight in the dark, filtered through a 0.45 µm PVDF membrane and finally made up to volume with Milli-Q water. Stock solutions were stored in the dark at 4 °C. Standard solutions of metal-chelates with concentrations lower than 100 µM were prepared daily from the stocks.

2.3 Plant materials

*o,o*EDDHA-free plant materials obtained from Fe-deficient and Fe-sufficient plants were used to develop the *o,o*EDDHA extraction procedure. Also, Fe-deficient plants were treated with Fe(III)-*o,o*EDDHA as described below to obtain tissues and plant fluids containing *o,o*EDDHA.

Sugar beet (*Beta vulgaris* L. cv. 'Orbis') and tomato (*Lycopersicon esculentum* L. cv. 'Tres Cantos') plants were grown in a growth chamber with a photosynthetic photon flux density (PPFD) of 350 µmol m^{-2} s^{-1} photosynthetically active radiation at leaf level, a photoperiod of 16 h light / 8 h dark, a temperature of 23 ºC during the day and 18 ºC during the night, and 80% relative humidity. Seeds were germinated and grown in vermiculite for 2 weeks. Seedlings were grown for 2 more weeks in half-strength Hoagland nutrient solution with 45 µM Fe(III)-EDTA at pH 5.5 and then transplanted to plastic buckets containing half-strength Hoagland nutrient solution with either 0 (*o,o*EDDHA-free Fe-deficient plants) or 45 µM Fe(III)-EDTA (*o,o*EDDHA-free Fe-sufficient plants). Some Fe-deficient plants were treated with a Fe(III)-EDDHA commercial fertilizer (90 µM Fe(III)-*o,o*EDDHA) in the nutrient solution for 24 hours. Nutrient solutions were buffered at pH values of approximately 7.0 for solutions without Fe and 5.5 for Fe-containing solutions. Leaves, roots and xylem sap were sampled from 42 day-old sugar beet and 35 day-old tomato plants. Xylem sap was sampled in sugar beet and tomato plants by centrifugation of petioles[27] and plant detopping,[28] respectively, and then filtered through 0.45 µm PVDF membranes and stored at -20 ºC until analysis. Plant tissues were washed with distilled water, frozen in liquid N_2 and stored at -20 °C until analysis.

Healthy peach (*Prunus persica* L. cv. 'Andros' and 'Babygold7') trees, not treated with Fe(III)-*o,o*EDDHA fertilizers in the full growing season, were sampled in the field in Zaragoza (Spain). Leaves were sampled 2-4 weeks after full bloom and fruits were harvested at commercial maturity dates. Leaves were washed with distilled water, frozen in liquid N_2 and stored at -20 °C until analysis. Fruits were peeled, a portion of the mesocarp was sampled from each opposite face and diced into 1 cm^3 pieces, and a composite sample

was built by mixing all of the small pieces from different peach fruits, immediately frozen in liquid N_2 and stored at -80 °C until analysis.[29]

2.4 Extraction of *o,o*EDDHA from plant materials

An extraction procedure suitable for subsequent HPLC-ESI/MS analysis was developed by modifying the procedure described by Bienfait *et al.*[9] An IS was always added (see below) to control possible systematic errors occurring during extraction, chromatography separation and ESI-ionization.

Frozen plant tissue (2.0-0.5 g of FW) was ground with 1-2 mL of extraction solution (1 mM ammonium acetate, pH 6.0) containing the IS, in a ZrO_2-ball mill (MM301, Retsch, Haan, Germany) operating at a frequency of 30 rps^{-1} until a homogeneous extract was obtained (approximate grinding times were 0.5, 1 and 2 min for fruit, leaf and root materials, respectively). The suspension was centrifuged at 12000 *g* for 20 min at 4 ºC and the supernatant was collected. These frequency and grinding time values were optimal to obtain a good separation between pellet and supernatant. Then, the pellet was resuspended in 1 mL of extraction solution, centrifuged again and the supernatant was collected and combined with the previous one. The last step was repeated once. The combined final extract was filtered through 0.45 µm PVDF membranes and made up to volume with extraction solution.

2.5 HPLC/ESI-MS(TOF) analysis

*o,o*EDDHA was determined as Fe(III)-*o,o*EDDHA using the HPLC/ESI-MS(TOF) method developed by Álvarez-Fernández *et al.*[14] Analyses were carried out with a Waters Alliance 2795 HPLC system (Waters, Mildford, MA, USA) coupled to a MicrOTOF (Bruker Daltonics, Bremen, Germany) TOF mass spectrometer equipped with an ESI ionization source. The MicrOTOF was operated in negative ion mode with endplate and spray tip potentials of 0.5 and 3.0 kV, respectively. Nebulizer gas (N_2) pressure and drying gas (N_2) flow rate were kept at 1.6 bar and 8.0 mL min^{-1} respectively, orifice voltage was 120 V and drying gas temperature was 180 ºC. Spectra were acquired in the 100-800 mass/charge ratio *(m/z)* range. The mass axis was calibrated using Li-formate adducts (10 mM LiOH, 0.2% (v/v) formic acid and 50% (v/v) 2-propanol).

Samples were separated by HPLC using a 5 µm particle size, 2.1 x 150 mm Waters Symmetry C_{18} column protected by a 3.5 µm particle size, 2.1 x 10 mm Waters Symmetry C_{18} guard column and a gradient of methanol and milli-Q water. pH was buffered in the chromatographic run with 5% of 20 mM ammonium acetate (pH 6.0). Injection volume and flow rate were 50 µL and 100 µl min^{-1}, respectively. Autosampler and column temperatures were 6 and 30 °C, respectively. The flow of the first 8 min of the chromatographic run was discarded by using a Reodhyne divert valve, in order to reduce ion suppression. The system was controlled with the software packages MicrOTOF Control version 2.2 (Bruker Daltonics) and HyStar version 3.2 (Bruker Daltonics). Data were processed with Data Analysis version 3.4 software (Bruker Daltonics). Concentrations of Fe(III)-*o,o*EDDHA were always quantified by external calibration with internal standardization.

2.6 Internal standards

Two types of internal standards (IS) were tested: i) two Fe stable isotope labelled (SIL) Fe(III)-*o,o*EDDHA analogues, ^{54}Fe(III)-*o,o*EDDHA and ^{57}Fe(III)-*o,o*EDDHA, and ii) a structural Fe(III)-*o,o*EDDHA analogue, Fe(III)-*o,o*EDDHMA. Iron(III)-*o,o*EDDHMA differs from Fe(III)-*o,o*EDDHA in the presence of two methyl groups located in both aromatic rings in *para* position (Fig. 4-1A,D).

Pre- and/or post-extraction IS addition techniques were applied as described by Taylor[21] using *o,o*EDDHA-free plant material. Pure standard solutions used for spiking samples were analyzed throughout the process as indicated below for the tissue samples. *o,o*EDDHA-free plant tissue extracts were first analyzed by HPLC/ESI-MS to ensure the absence of endogenous isobaric compounds co-eluting with the analyte and/or the IS that could cause analytical interferences. When using as SIL ^{54}Fe(III)-*o,o*EDDHA, two pre-extraction assays were carried out by spiking directly plant material before the extraction process with the analyte (^{nat}Fe(III)-*o,o*EDDHA) and/or the IS (^{54}Fe(III)-*o,o*EDDHA). Plant tissue to final extract weight ratios (PTW/FEW ratios, in w/w) were 160 mg FW g^{-1}, resulting in final analyte and IS concentrations in the range of 15 to 35 µM. For the first assay, a spike of a combined ^{nat}Fe(III)-*o,o*EDDHA plus ^{54}Fe(III)-*o,o*EDDHA solution was added to leaves and roots from Fe-sufficient sugar beet and tomato plants, then the extraction process was carried out as described above and the extracts were analyzed immediately by HPLC/ESI-MS. A second different pre-extraction assay was carried out by spiking only ^{54}Fe(III)-*o,o*EDDHA into leaves, roots and xylem sap from Fe-deficient and Fe-sufficient sugar beet plants. Leaf and root extracts were obtained, left at 4 ºC in darkness and analyzed 0, 1, 3, 6, 12 and 24 hours later. Xylem sap samples were left at 4 ºC in darkness and analyzed 0, 1, 3, 6, 12 and 24 hours after the spiking process.

When using the structural analogue Fe(III)-*o,o*EDDHMA, post- and pre-extraction addition assays were applied to six different plant tissues from Fe-sufficient plants: leaves and roots from sugar beet and tomato plants and leaves and fruits from peach trees. The final analyte and IS concentrations were approximately 16 and 8 µM, respectively. First, a post-extraction assay was carried out by spiking plant tissues after the extraction process with both the analyte and the IS, and the extract was then immediately analyzed by HPLC/ESI-MS. Different PTW/FEW (w/w) ratios (60, 80, 120, 160 and 250 mg FW g^{-1} extract) were used with each plant tissue. Second, a pre-extraction assay was carried out by spiking directly plant tissues before the extraction process with both the analyte and the IS. The extraction process was carried out at the optimum plant PTW/FEW ratio for each plant tissue as determined in the post-extraction assay. When using xylem sap from tomato and sugar beet, the sample was spiked directly with both the analyte and the IS just before the HPLC/ESI-MS analysis.

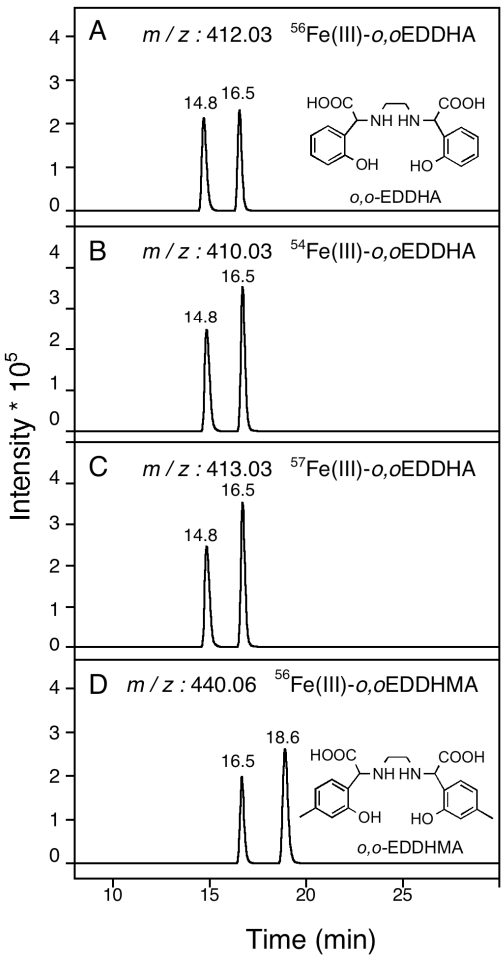

Figure 4-1. Chromatograms extracted at the *m/z* values of the major $[M - H]^{-1}$ molecular ions of the analyte ^{nat}Fe(III)-*o,o*EDDHA (^{56}Fe(III)-*o,o*EDDHA; A), the compounds tested as SIL-IS (^{54}Fe(III)-*o,o*EDDHA and ^{57}Fe(III)-*o,o*EDDHA; B-C), and the IS structural analogue ^{nat}Fe(III)-*o,o*EDDHMA (^{56}Fe(III)-*o,o*EDDHMA; D) at a concentration of 20 µM. The first and second peaks of the Fe(III)-*o,o*EDDHA chromatograms correspond to the racemic and meso isomers, respectively. Solutions were 1 mM ammonium acetate, pH 6.0.

2.7 Recovery assays and limits of detection and quantification

Two recovery assays were also carried out with *o,o*EDDHA-free Fe-sufficient plant materials (leaves and roots from sugar beet and tomato plants and leaves and fruits from peach trees). This was carried out by adding, before the extraction process, known amounts of either a mixture of Fe(III)-*o,o*EDDHA plus the IS Fe(III)-*o,o*EDDHMA or a combined solution of different metal-*o,o*EDDHA chelates (Ni(II)-, Co(II)-, Zn(II)-, Mn(II)-, Mg-, Ca- and Cu(II)-*o,o*EDDHA) plus the IS (Fe(III)-*o,o*EDDHMA). An aliquot of the extract obtained as described above was treated with NH_4OH until pH 11-12 to dissociate the metal-*o,o*EDDHA complexes, an excess of $FeCl_3$ was slowly added and the pH was adjusted to 6.0–7.0 with HCl to form Fe(III)-*o,o*EDDHA. Then, the solution was filtered through 0.45 µm PVDF membranes and made up to volume with extraction solution at the final PTW/FEW ratios found optimal for each plant tissue (see Table 4-1). Final concentrations of the analyte and IS were 20 and 10 µM, respectively. For xylem sap, the

recovery assays were carried out only with tomato, by directly spiking both the analyte and the IS before NH_4OH addition and using the same protocol described above, while keeping minimal the dilution of the sample (PTW/FEW ratios of approximately 500 mg FW g^{-1}). All samples were immediately analyzed by HPLC/ESI-MS.

Detection (LODs) and quantification (LOQs) limits are defined as the amount of analyte necessary per g (FW) of plant tissue to give a signal/noise (S/N) ratio of 3 and 10, respectively.

2.8 Analysis of plant materials treated with Fe(III)-*o,o*EDDHA

Samples of leaves (1 g FW), roots (2 g FW) and xylem sap (0.3 g FW, approximately 300 µL) from sugar beet and tomato plants treated with Fe(III)-*o,o*EDDHA were spiked directly with the IS Fe(III)-*o,o*EDDHMA. Tissues were extracted according to the extraction procedure described above, and then made up to 5 g final weight with extraction solution. Tissue extracts (2 g aliquots) and xylem sap samples (approximately 0.3 g aliquots) were treated with NH_4OH (25% v/v) until pH 11.0-12.0, an excess of $FeCl_3$ (2.5 µmol) was slowly added and the pH was adjusted to 6.0-7.0 with HCl. Then the solution was filtered through 0.45 µm PVDF membranes and made up to 5 g final weight for tissue extracts and to approximately 600 mg for xylem sap samples, using extraction solution. Final concentration of the IS in these solutions was 1 µM. *Racemic* and *meso–o,o*EDDHA were determined as their Fe(III)-chelate forms by HPLC/ESI-MS[14] using calibration curves corrected with the internal standardization procedure described above.

3. RESULTS AND DISCUSSION

Matrix effects for both Fe(III)-*o,o*EDDHA isomers (*racemic* and *meso*) were evaluated for all plant tissues at different plant tissue to final extract weight ratios (PTW/FEW). Strong matrix effects during the ionization of the analyte were found, with ion suppression occurring in most cases, although tomato tissues were an exception. Ion suppression accounted for approximately between 10 and 40% of the analyte signal, the largest values being found for peach tissues. Matrix effects were very similar for both *racemic* and *meso* isomers and were less marked when PTW/FEW values were lower (data not shown). In some materials such as tomato tissues, matrix effects were only minor. Since the use of IS is considered the most appropriate method for controlling matrix effects during LC/ESI-MS experiments, several IS were tested in subsequent experiments.

3.1 Internal standards

3.1.1 Using Fe stable isotope-labelled Fe(III)-o,oEDDHA as internal standards

LC/ESI-MS(TOF) ion chromatograms of the analyte and the SIL molecules tested as IS were extracted at *m/z* values of the corresponding $[M - H]^{-1}$ molecular ions (412.0 for ^{56}Fe(III)-*o,o*EDDHA, 413.0 for ^{57}Fe(III)-*o,o*EDDHA and 410.0 for ^{54}Fe(III)-*o,o*EDDHA), with a precision of ± 0.02 *m/z* (Fig. 4-1A-C). Fe(III)-*o,o*EDDHA geometric isomers were separated by HPLC, with elution times for the *racemic* and *meso* isomers of 14.8 and 16.5 min, respectively.

Plant tissue samples free of *o,o*EDDHA were also analyzed by HPLC/ESI-MS to look for possible isobaric compounds co-eluting with the analytes and/or the IS that may interfere with the determination. An endogenous isobaric interference at *m/z* 413.0 was found to co-elute partially with *meso* ^{57}Fe(III)-*o,o*EDDHA in sugar beet materials, whereas no isobaric interferences were found in all other cases (data not shown). Therefore, ^{57}Fe(III)-*o,o*EDDHA was discarded as a possible SIL, and only ^{54}Fe(III)-*o,o*EDDHA was used as SIL internal standard in further experiments.

A pre-extraction assay was carried out by spiking a combined ^{nat}Fe(III)-*o,o*EDDHA plus ^{54}Fe(III)-*o,o*EDDHA solution into sugar beet and tomato plant tissues before the extraction process, and *racemic* and *meso* Fe(III)-*o,o*EDDHA were quantified by HPLC/ESI-MS. Recoveries were calculated for each isomer by dividing the concentration found in the extract by the concentration found in the pure combined standard solution used to spike the samples. Recoveries found were in the range of 150-180% for all plant materials, indicating that the use of the IS ^{54}Fe(III)-*o,o*EDDHA did not give satisfactory results. Problems are unlikely to occur during HPLC/ESI-MS analysis, since ^{nat}Fe(III)-*o,o*EDDHA and ^{54}Fe(III)-*o,o*EDDHA co-elute in pure solutions (Fig. 4-1) and plant extracts (not shown). A likely cause for the >150% recovery values found in the spiked plant materials is the occurrence of Fe isotope exchange reactions between the ^{54}Fe originally bound to *o,o*EDDHA and the added ^{nat}Fe(III)-*o,o*EDDHA and native Fe present in plant materials, that could result in the *de novo* formation of ^{nat}Fe(III)-*o,o*EDDHA, the breakdown of ^{54}Fe(III)-*o,o*EDDHA or both (Eqn (1)). The equilibrium could be shifted towards the formation of ^{nat}Fe(III)-*o,o*EDDHA because of the higher concentration of ^{nat}Fe-compounds present in plant materials compared with ^{54}Fe bound to *o,o*EDDHA.

$$^{54}\mathrm{Fe(III)}-o,o\mathrm{EDDHA} + {}^{nat}\mathrm{Fe-plant} \Leftrightarrow {}^{nat}\mathrm{Fe(III)}-o,o\mathrm{EDDHA} + {}^{54}\mathrm{Fe-plant} \qquad (1)$$

The occurrence of isotope exchange reactions was assessed by using a second pre-extraction addition assay, spiking only ^{54}Fe(III)-*o,o*EDDHA into sugar beet plant materials, and then monitoring the analyte to IS peak area ratio (As/AIS; ^{56}Fe(III)-*o,o*EDDHA/^{54}Fe(III)-*o,o*EDDHA) for the next 24 hours (Fig. 4-2). The As/AIS values for each isomer, plant material and analysis time were compared with those of a pure standard solution (0.06). Isotope exchange (As/AIS values higher than 0.06) occurred in many cases, depending on the type of plant material used and the specific isomer (Fig. 4-2). For leaf and root extracts, the As/AIS ratio was significantly larger than 0.06 for both isomers (Fig. 4-2A-D). No dependence of the As/AIS with the time elapsed between extraction and analysis was observed, indicating that isotope exchange reactions occurred mainly during the extraction process. The plant Fe nutritional status also affected markedly the extent of isotope exchange reactions, since the As/AIS ratios obtained for Fe-sufficient leaves (0.4-0.5) and roots (6.0-8.0) were significantly higher (at $p<0.05$) than those corresponding to Fe-deficient tissues (values of 0.1-0.2 for leaves and 0.1-0.3 for roots). Iron concentrations in Fe-sufficient roots were 8-fold higher than those of Fe-deficient, whereas Fe-sufficient leaves had Fe concentrations only 3-fold higher than those of Fe-deficient ones.

In xylem sap, and in contrast to what happened in leaves and roots, only the less stable isomer (*meso* ^{54}Fe(III)-*o,o*EDDHA; log K 34.15)[30] appeared to participate in Fe isotope exchange reactions, whereas the most stable isomer (*racemic* ^{54}Fe(III)-*o,o*EDDHA; log K 35.86)[30] did not. This could be due to an effect of the different endogenous Fe/spiked ^{54}Fe ratios occurring in leaf and root extracts and xylem sap samples, which were in the ranges of 1.2-1.5 in leaf and root extracts, and 0.2-0.5 in xylem sap. Therefore, the amount of native Fe in xylem sap was likely limiting for isotope exchange reactions, favoring isotopic exchange only with the less stable isomer. Time-dependence of the Fe isotope exchange

reactions of *meso* ^{54}Fe(III)-*o,o*EDDHA was observed in the xylem sap of both Fe-deficient and -sufficient plants (Fig. 4-2 E-F), since the As/AIS ratio increased with time. At time 0 h (analyzed immediately after spiking) very little, if any, isotope exchange reactions had occurred, whereas significant increases in the As/AIS ratio occur mainly before 6 and 12 hours in Fe-deficient and Fe-sufficient samples, respectively. Isotope exchange reactions occurring in xylem sap were favored in Fe-deficient extracts compared with Fe-sufficient ones, in spite of the fact that Fe concentrations were lower in Fe-deficient than in Fe-sufficient xylem sap (6 and 10 µM Fe, respectively). This suggests that endogenous Fe-compounds present in Fe-deficient xylem sap undergo Fe isotope exchange reactions more easily than those present in Fe-sufficient xylem, most likely due to the significant differences occurring in chemical composition.[27]

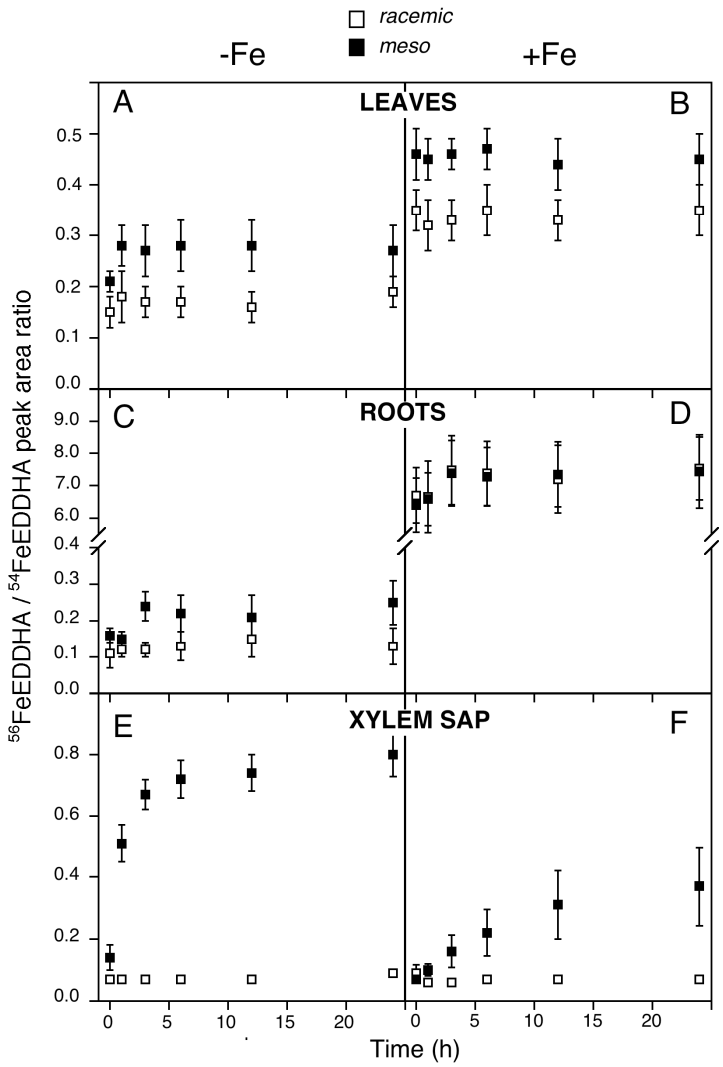

Figure 4-2. Time dependence of the ^{56}Fe(III)-*o,o*EDDHA/^{54}Fe(III)-*o,o*EDDHA ratios in a pre-extraction addition assay carried out with extracts of Fe-deficient (-Fe) and Fe-sufficient (+Fe) sugar beet leaves (A, B) and roots (C, D) and with xylem sap (E, F). Plant tissues were spiked only with ^{54}Fe(III)-*o,o*EDDHA and analyzed at different times after the extraction (leaves and roots) or the spiking process (xylem sap). Open squares are for the *racemic* isomer and full squares for the *meso* one. Data are means ± SE (n = 3).

These results support that Fe SIL compounds are not suitable as IS for the quantification of Fe(III)-*o,o*EDDHA in many plant materials. However, they could be adequate for the quantification of different synthetic Fe(III)-chelates (including Fe(III)-*o,o*EDDHA) in liquid agricultural matrices such as soil solutions, irrigation waters, nutrient solutions and even in some plant samples such as peach xylem sap.[14] Although SIL compounds are generally supposed to be the best IS, the results of the present study indicate that they must be tested for each specific matrix. Isotope exchange reactions between SIL compounds used as IS have been also described in previous studies. Chavez *et al.* found isotope exchange reactions between the D atoms of the rofecobix SIL $^{13}CD_3$rofecoxib and the ^{1}H atoms of the water-containing LC solvents when they were analyzed by HPLC/MS, but not when non-polar solvents were used in GC/MS analysis.[25] In that study, a ^{13}C-labeled compound, $^{13}C_7$rofecoxib, was successfully used, concluding that ^{13}C-, ^{15}N- or ^{17}O- SIL analogues may better choices than D-labelled compounds. However, these SIL compounds are not always commercially available or they are very expensive, as it occurs in the case of *o,o*EDDHA (approximately 3000 euros per mg). In these cases, structural analogues can be an alternative to SIL internal standards, taking into consideration structural similarities between the IS and the analyte. [22, 31]

3.1.2 *Using the structural analogue Fe(III)-o,oEDDHMA as internal standard*

In our case, the compound Fe(III)-*o,o*EDDHMA was considered as the best option for a IS structural analog of Fe(III)-*o,o*EDDHA, because of the high chemical similarity, the only differences being the two methyl groups located in *para* position (Fig. 4-1A,D). The compound Fe(III)-*o,o*EDDHMA is also accepted by EU legislation as a commercial fertilizer, but its use is much less frequent than that of Fe(III)-*o,o*EDDHA (3 and 72% of the commercial products in Spain in 2007, respectively).[32] Therefore, it would be necessary to ascertain that Fe(III)-*o,o*EDDHMA is not present in plant samples prior to apply the proposed *o,o*EDDHA analysis method. A LC/ESI-MS(TOF) ion chromatogram of Fe(III)-*o,o*EDDHMA is shown in Fig. 4-1D. The ion chromatogram was extracted at the corresponding ^{56}Fe isotope signal of the $[M - H]^{-1}$ molecular ion at a *m/z* value of 440.0. Retention times were 16.5 min and 18.6 min for isomer 1 and isomer 2 of Fe(III)-*o,o*EDDHMA, respectively (Fig. 4-1D). Isomer 1 of Fe(III)-*o,o*EDDHMA co-eluted with the *meso* isomer of the analyte, and therefore it was chosen to be tested as IS. Since the *racemic* Fe(III)-*o,o*EDDHA isomer eluted at a different time than the IS, the possibility that matrix effects associated to the presence of other co-eluting compounds may occur should be also assessed.

The post-extraction assay previously used by other authors[12,33-35] was carried out by spiking a combined ^{nat}Fe(III)-*o,o*EDDHA plus ^{nat}Fe(III)-*o,o*EDDHMA solution into plant extracts before the HPLC/ESI-MS analysis. Different PTW/FEW ratios were tested for each plant material, including sugar beet and tomato leaves and roots and peach leaves and fruits. PTW/FEW ratios tested were similar or lower than those used by Bienfait *et al.*[9] Recoveries were calculated as described above (Table 4-1). For all plant materials, recoveries of *racemic* Fe(III)-*o,o*EDDHA were affected by the PTW/FEW ratio (Table 4-1). For high PTW/FEW ratios, recoveries were in the range of 119-109%, which indicated a slight increase of the analyte/IS response in comparison with the pure standard solution. Therefore, the co-eluting matrix components caused either an enhancement of the analyte ionization or a suppression of the IS ionization. With the lowest PTW/FEW ratios used with each material, recovery values were good, in the range of 96-104%, indicating that

matrix effects affected in a similar way analyte and IS. Optimal PTW/FEW ratio values (in mg per g) were 80 for sugar beet and tomato leaves, 120 for peach leaves, 160 for roots and 60 for peach fruits.

Table 4-1. Recoveries (in %) of *racemic* and *meso* Fe(III)-*o,o*EDDHA in the post-extraction assay, using Fe(III)-*o,o*EDDHMA as IS. Six different plant tissues were used at different plant tissue/final extract weight ratios (PTW/FEW, w/w). Data are means ± SE (n = 3).

Plant tissue ratio (PTW/FEW, mg FW g^{-1})	*racemic*	*meso*
Sugar beet leaves		
160	110 ± 4	97 ± 1
120	111 ± 2	99 ± 1
80*	103 ± 2	98 ± 1
Sugar beet roots		
250	117 ± 4	105 ± 1
160*	102 ± 2	103 ± 1
Tomato leaves		
160	114 ± 4	98 ± 1
120	110 ± 1	97 ± 2
80*	103 ± 2	96 ± 2
Tomato roots		
250	114 ± 2	104 ± 1
160*	102 ± 2	98 ± 1
Peach leaves		
160	109 ± 2	98 ± 3
120*	99 ± 2	102 ± 1
Peach fruits		
160	117 ± 2	113 ± 1
120	119 ± 2	103 ± 1
80	113 ± 1	104 ± 3
60*	104 ± 2	101 ± 1

*Optimal PTW/FEW ratios.

Using these optimal PTW/FEW ratios, a pre-extraction assay was carried out to test the suitability of the structural analogue Fe(III)-*o,o*EDDHMA as IS in the whole analytical method, including the extraction process, by spiking directly plant materials before the extraction process with a combined Fe(III)-*o,o*EDDHA plus Fe(III)-*o,o*EDDHMA solution. Good recoveries, in the range of 93-106% for *racemic* Fe(III)-*o,o*EDDHA and 96-105% for *meso* Fe(III)-*o,o*EDDHA, were obtained for all plant materials (Table 4-2). In summary, the structural analogue Fe(III)-*o,o*EDDHMA could be considered as a suitable IS for the determination of Fe(III)-*o,o*EDDHA in plant tissue extracts.

Table 4-2. Recoveries (in %) of *racemic* and *meso* Fe(III)-*o,o*EDDHA in the pre-extraction assay, using Fe(III)-*o,o*EDDHMA as IS. Eight different plant tissues were used at the optimal PTW/FEW ratios found in the post-extraction assay (see Table 4-1). Data are means ± SE (n = 5).

		racemic	*meso*
Sugar beet	Leaves	95 ± 3	100 ± 1
	Roots	100 ± 2	101 ± 5
	Xylem sap	96 ± 1	99 ± 1
Tomato	Leaves	93 ± 4	100 ± 1
	Roots	101 ± 2	103 ± 2
	Xylem sap	95 ± 2	96 ± 1
Peach	Leaves	100 ± 4	105 ± 2
	Fruits	106 ± 2	101 ± 1

3.2 Recovery assays

It is assumed that the chelating agent *o,o*EDDHA could occur inside the plant in chemical forms other than Fe(III)-*o,o*EDDHA.[36] Therefore, to ensure the total *o,o*EDDHA determination in plant materials, recovery assays were also carried out by adding an excess of Fe(III) to the plant extracts to facilitate the chelation of all *o,o*EDDHA chemical forms with Fe(III), and its subsequent determination as Fe(III)-*o,o*EDDHA by HPLC/ESI-MS. The pH of plant extracts were adjusted to pH 11-12 before the addition of Fe excess to facilitate the existence of free, soluble *o,o*EDDHA forms, and then the pH was slowly adjusted to neutral values, where Fe(III)-*o,o*EDDHA is known to be stable.[30]

Two different pre-extraction recovery assays were carried out by spiking plant tissues with either Fe(III)-*o,o*EDDHA plus the IS Fe(III)-*o,o*EDDHMA or a combined standard solution of other metal-*o,o*EDDHA complexes plus the IS Fe(III)-*o,o*EDDHMA. For both assays, recoveries were similar and acceptable (in the range of 74-118%) independently of the metal-*o,o*EDDHA complex spiked into the plant material (Table 4-3). Differences between isomers were observed, with *meso* recovery values being better (average of 104%) than those of *racemic* (average of 90%), as also observed in the previous assays (Tables 4-1 and 4-2). The reason for that is likely due to the fact that the IS co-eluted with the *meso* Fe(III)-*o,o*EDDHA isomer, whereas the *racemic* Fe(III)-*o,o*EDDHA isomer had a different retention time. When comparing plant materials, the lowest recoveries were obtained for the *racemic* isomer in tomato leaves (79%) and roots (77%). Since the protocol of these assays was more complex than those used in previous assays (Tables 4-1 and 4-2), involving several steps and including pH changes, higher standard errors were obtained (Table 4-3).

Table 4-3. Recoveries (in %) of *racemic* and *meso o,o*EDDHA in a pre-extraction assay in the presence of an Fe excess, using Fe(III)-*o,o*EDDHMA as IS, after spiking plant tissues with Fe(III)-*o,o*EDDHA (Assay 1) or a combined solution of Ca(II)-, Mg(II)-, Cu(II)-, Mn(II)-, Ni(II)-, Co(II)-, Zn(II)-*o,o*EDDHA (Assay 2). Data are means ± SE (n = 5).

		Assay 1		Assay 2	
		racemic	*meso*	*racemic*	*meso*
Sugar beet	Leaves	110 ± 4	104 ± 2	101 ± 6	97 ± 2
	Roots	94 ± 6	106 ± 8	86 ± 6	111 ± 6
Tomato	Leaves	83 ± 3	107 ± 2	75 ± 4	98 ± 1
	Roots	80 ± 1	103 ± 2	74 ± 2	95 ± 2
	Xylem sap	103 ± 7	108 ± 4	90 ± 2	90 ± 5
Peach	Leaves	93 ± 6	116 ± 10	90 ± 8	118 ± 5
	Fruits	93 ± 2	102 ± 1	84 ± 1	95 ± 2

3.3 Limits of detection and quantification

LODs and LOQs for *racemic* and *meso o,o*EDDHA in different plant materials are shown in Table 4-4. Overall sensitivity was high, with LOQs between 11 and 162 pmol g^{-1} FW for plant tissues and between 60 and 151 pmol mL^{-1} for xylem sap. These values are at least an order of magnitude better than the only LOQ reported so far, that was 2140 pmol g^{-1} FW for tomato, lettuce and pepper using HPLC/UV-VIS.[9] In other studies with tobacco and tomato, LOQs were not reported but the minimum amount of Fe(III)-*o,o*EDDHA determined was also very high, 3500 pmol Fe(III)-*o,o*EDDHA g^{-1} FW.[8]

Table 4-4. Limits of detection (LOD) and quantification (LOQ) for *racemic* and *meso* Fe(III)-*o,o*EDDHA in eight different plant tissues. LOD and LOQ values were in pmol g^{-1} FW for plant tissues and in pmol mL^{-1} for xylem sap.

		LOD		LOQ	
		racemic	*meso*	*racemic*	*meso*
Sugar beet	Leaves	24	18	81	61
	Roots	9	8	30	27
	Xylem sap	26	18	87	60
Tomato	Leaves	9	10	29	34
	Roots	3	4	11	14
	Xylem sap	45	35	151	118
Peach	Leaves	49	29	162	98
	Fruits	12	12	38	41

3.4 Analysis of *o,o*EDDHA in plant materials

Fe-deficient sugar beet and tomato plants were treated with a Fe(III)-EDDHA commercial fertilizer (90 µM Fe(III)-*o,o*EDDHA) for 24 h. Leaves, roots and xylem sap were sampled and submitted to the extraction method developed, using as IS Fe(III)-*o,o*EDDHMA. HPLC/ESI-MS(TOF) analysis of the extracts were performed before and after the addition of an Fe excess to determine the *o,o*EDDHA bound to Fe(III) and the sum

of all *o,o*EDDHA chemical forms occurring in the extracts, respectively. Ion chromatograms showing the occurrence of Fe(III)-*o,o*EDDHA in tomato leaf and root extracts and xylem samples are shown in Fig. 4-3. *Racemic* and *meso* Fe(III)-*o,o*EDDHA were found at 14.8 and 16.5 min, respectively; a third peak was found at 14.8 min and was identified as the Fe(III)-*o,p*EDDHA isomer[14] that is also present in the commercial Fe(III)-EDDHA fertilizer used (this product contains approximately 4.3 and 0.9 g of Fe(III)-*o,o*EDDHA and Fe(III)-*o,p*EDDHA per 100 g of product, respectively).[14]

Both *o,o*EDDHA and Fe(III)-*o,p*EDDHA were found in all plant materials in both plant species (Table 4-5). *o,o*EDDHA was mainly in the Fe(III)-chelate form in leaves, roots (approximately 67% in both cases) and in xylem sap (approximately 90%).

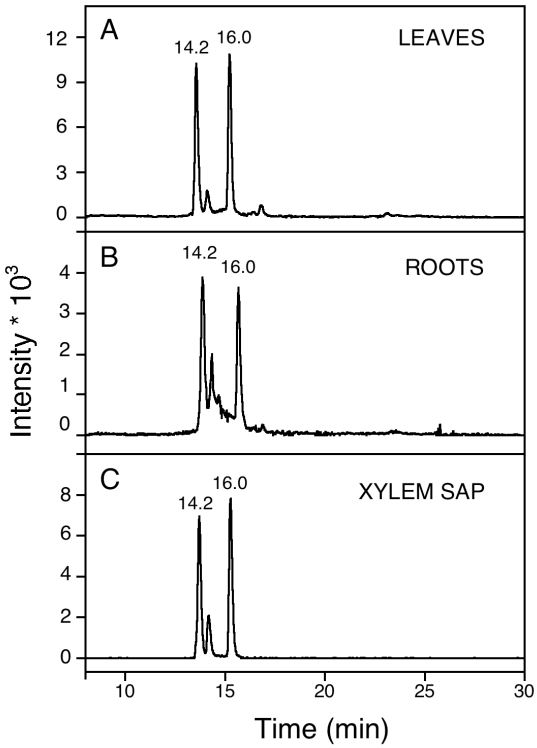

Figure 4-3. Ion chromatograms (at *m/z* 412.0) for extracts of leaves (A) and roots (B), and xylem sap (C) from tomato plants treated with a commercial Fe(III)-EDDHA fertilizer for 24 hours. *Racemic* and *meso* Fe(III)-*o,o*EDDHA were found at 14.2 and 16.0 min respectively. The peak at 14.8 min retention time corresponded to Fe(III)-*o,p*EDDHA.

The presence of *o,o*EDDHA in plants was first demonstrated in leaves[8] by direct VIS spectophotometry of purified extracts. More recently, *o,o*EDDHA was found in leaves and roots by HPLC/VIS.[9] In our study we found leaf and root *o,o*EDDHA concentrations in the range of 12-63 nmol g^{-1} FW with a short exposure time to Fe(III)-*o,o*EDDHA, whereas in previous studies[8,9], carried out in many cases with longer exposure times, concentrations were in the range 7-30 nmol g^{-1} FW. In the present study, *o,o*EDDHA concentrations in leaves were always 2-4 fold higher than those of roots in sugar beet and tomato, whereas previous studies reported similar values in roots and leaves.[9] Both isomers occurred in similar concentrations in all tissues and in both species, with *racemic*/*meso* ratios in the range of 0.9-1.2. However, lower values for that ratio were found in pepper and tomato

leaves (0.7-1.0), whereas in roots ratios were of approximately 1, and this was attributed to differences in translocation from roots to shoots or/and in degradation in leaves between isomers.[9] Prolonged treatments such as those used by Bienfait *et al.* apparently seem to facilitate a preferential occurrence of the *meso* isomer.[9]

Table 4-5. Fe(III)-*o,o*EDDHA and *o,o*EDDHA concentrations (in nmol g^{-1} FW for plant tissues and in nmol mL^{-1} for xylem sap) in plants treated with a Fe(III)-EDDHA commercial fertilizer (90 µM Fe(III)-*o,o*EDDHA) for 24 h. Data are means ± SE (n = 3).

		racemic	*meso*	Total
		Fe(III)-*o,o*EDDHA concentrations		
Sugar beet	Leaves	8.5 ± 0.5	7.4 ± 0.5	15.9 ± 1.0
	Roots	4.3 ± 0.4	3.7 ± 0.2	8.0 ± 0.4
	Xylem sap	0.16 ± 0.01	0.15 ± 0.01	0.31 ± 0.01
Tomato	Leaves	19.9 ± 0.8	20.3 ± 0.8	40.2 ± 0.0
	Roots	6.4 ± 1.2	5.2 ± 0.8	11.5 ± 2.0
	Xylem sap	0.5 ± 0.2	0.4 ± 0.2	0.9 ± 0.4
		*o,o*EDDHA concentrations		
Sugar beet	Leaves	11.9 ± 0.9	11.7 ± 0.8	23.6 ± 1.8
	Roots	6.3 ± 0.8	5.8 ± 0.8	12.1 ± 1.6
Tomato	Leaves	30.0 ± 3.0	33.0 ± 2.9	63.0 ± 5.8
	Roots	9.5 ± 1.6	8.5 ± 1.3	18.0 ± 2.9
	Xylem sap	0.6 ± 0.1	0.5 ± 0.1	1.1 ± 0.1

Results show for the first time that Fe(III)-*o,p*EDDHA is also present in leaves, roots and xylem sap of plants treated with commercial Fe(III)-EDDHA products (Fig. 4-3). However, quantification was not possible because of the lack of commercially available standards. Further research is needed to design and validate an appropriate methodology to determine this compound.

4. CONCLUSIONS

The method developed permits the determination by HPLC/ESI-MS of the xenobiotic *o,o*EDDHA chelating agent used in Fe-fertilizers, with extreme selectivity, high sensitivity and sufficient accuracy and reproducibility, in a wide range of species and plant tissues. Samples tested include sugar beet leaves and roots, tomato leaves and roots and peach leaves and fruits. The results presented in this paper demonstrate the need for a careful evaluation and proper choice of the IS used for quantification in complex matrices such as plant materials, when using HPLC/ESI-MS based methods. Iron stable isotope labelled Fe-*o,o*EDDHA does not appear to be a suitable IS, mainly because of the occurrence of isotope exchange reactions during extraction and/or sample treatment. An adequate internal standard would probably be any ^{13}C-, ^{15}N- or ^{17}O- stable isotope labelled chelating agent (*o,o*EDDHA), but they are not commercially available. A structural analogue, one of the Fe(III)-*o,o*EDDHMA isomers, has been confirmed to be an adequate IS for *o,o*EDDHA determination in plant tissues by HPLC/ESI-MS, therefore constituting a useful tool for studies on *o,o*EDDHA plant uptake, transport and allocation. *o,o*EDDHA was found in all plant tissues tested in tomato and sugar beet plants treated with moderate (90 µM) Fe(III)-*o,o*EDDHA doses for only one day.

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Capítulo 5

RAPID COMMUNICATIONS IN MASS SPECTROMETRY

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ELECTROSPRAY COLLISION-INDUCED DISSOCIATION MASS SPECTROMETRY: A TOOL TO CHARACTERIZE SYNTHETIC POLYAMINOCARBOXYLATE FERRIC CHELATES USED AS FERTILIZERS

Irene Orera[a], Jesús Orduna[b], Javier Abadía[a] and Ana Álvarez-Fernández[a]

[a]Plant Nutrition Department, Estación Experimental de Aula Dei, Consejo Superior de Investigaciones Científicas (CSIC), Apdo. 202, 50080 Zaragoza, Spain.

[b]New Organic Materials Unit, Institute of Materials of Aragón, CSIC-University of Zaragoza, c/ Pedro Cerbuna 12, E-50009 Zaragoza, Spain.

ELECTROSPRAY COLLISION-INDUCED DISSOCIATION MASS SPECTROMETRY: A TOOL TO CHARACTERIZE SYNTHETIC POLYAMINOCARBOXYLATE FERRIC CHELATES USED AS FERTILIZERS.

Abstract: Fertilizers based on synthetic polyaminocarboxylate ferric chelates have been known to be successful in supplying Fe to plants since the 1950s. In commercial Fe(III)-chelate fertilizers, a significant part of the water-soluble Fe fraction consists on still uncharacterized Fe byproducts, whose agronomical value is unknown. Although collision-induced dissociation (CID) tandem mass spectrometry (MS/MS) is a valuable tool for identification, no fragmentation pattern data have been reported for most Fe(III)-chelate fertilizers so far. The aim of this study was to characterize the CID-MS^2 fragmentation patterns of the major synthetic Fe(III)-chelates used as Fe fertilizers, to subsequently use this technique for the characterization of commercial fertilizers. Quadrupole-time of flight (QTOF) and spherical ion trap mass analyzers equipped with an electrospray (ESI) ionization source were used. ESI-CID-MS^2 spectra obtained were richer when using the QTOF device. Specific differences were found among Fe(III)-chelate fragmentation patterns, even in the case of positional isomers. The analysis of a commercial Fe(III)-chelate fertilizer by high resolution liquid chromatography (HPLC) coupled to ESI-QTOF MS revealed two previously unknown, Fe-containing compounds, that were successfully identified by a comprehensive comparison of the ESI-CID-MS^2(QTOF) spectra with those of pure chelates. This shows that HPLC/ESI-CID-MS^2(QTOF), along with Fe(III)-chelate fragmentation patterns, could be a highly valuable tool to directly characterize the water-soluble Fe fraction in Fe(III)-chelate fertilizers. This could be of great importance in issues related to crop Fe fertilization, both from an agricultural and an environmental point of view.

Keywords: CID, iron fertilizers, mass spectrometry, mass fragmentation, synthetic ferric chelates

1. INTRODUCTION

Iron is an essential micronutrient for plants, required for important metabolic processes such as respiration, photosynthesis, nitrogen fixation and synthesis of DNA and hormones.[1] Iron deficiency (also called Fe chlorosis) is a widespread nutritional disorder that limits crop yields in many agricultural areas of the world. Since the incidence of Fe-deficiency in crops has increased markedly in the recent years[2] the use of Fe-fertilizers is greater than ever. The efficiency of Fe fertilizers derived from synthetic polyaminocarboxylate Fe(III)-chelates has been known since the 1950's. The application of these Fe(III)-chelates is considered as the most effective option to control Fe deficiency and, in spite of the high cost, these fertilizers are nowadays commonly used in soil-less horticulture as well as in high value field grown crops.[3]

The synthetic polyaminocarboxylate compounds used to produce Fe fertilizers are strong binding chelating agents from the ethylenediaminecarboxylic acid family, and include ethylenediamine tetraacetic acid (EDTA), diethylenetriamine pentaacetic acid (DTPA), N-(2-hydroxyethyl)ethylenediaminetriacetic acid (HEEDTA), cyclohexane-1,2-diaminetetraacetic acid (CDTA), ethylenediamine-N-N'bis(*o*-hydroxyphenylacetic) acid (*o*,*o*EDDHA), ethylenediamine-N-N'bis(2-hydroxy-4-methylphenylacetic) acid (EDDHMA), ethylenediamine-N-N'bis(5-carboxy-2-hydroxyphenylacetic) acid (EDDCHA) and ethylenediamine-N-N'bis-(2-hydroxy-5-sulphophenylacetic) acid (EDDHSA). All these compounds have high denticity (5 to 8 donor groups available for metal chelation), high affinity for Fe(III), and a structure that allows the formation of highly stable Fe(III)-chelate complexes *via* simultaneous coordination of several donor groups in a given chelating agent molecule to a single Fe(III) atom. Therefore, the most common coordination arrangement described for the chelation of Fe(III) by these chelating agents is a mononuclear Fe(III)-chelate complex with 1:1 stoichiometry, where Fe is generally found in a six-coordinate, roughly octahedral field, and the chelating agent coordinating as a sexadentate one.

In spite of the wide use of these fertilizers, the biological and environmental implications of this agronomical practice are still not fully known, with most of the studies being focused mainly on Fe(III)-EDTA and Fe(III)-DTPA. In fact, these compounds are under scrutiny due to their influence on metal availability and mobility, specially because of the persistence in the environment.[4,5] Parameters related to the efficacy of the synthetic Fe(III)-chelates as fertilizers are tightly regulated in Europe, with several legislation changes in the last years.[6-8] Regulated items include the list of authorized synthetic chelating agents, the minimal values for water-soluble Fe and total chelated Fe contents, and the Fe content chelated by each authorized chelating agent. In addition, official analytical methods based on atomic absorption spectroscopy (AAS) and high performance liquid chromatography (HPLC) coupled to UV-Vis, have been recently approved to determine the parameters specified by these regulations.[9-13] The lack of an unique official method for the simultaneous determination of all authorized synthetic Fe(III)-chelates has been mainly due to the low specificity of the detection techniques, which require a very good chromatographic separation, specially for compounds with similar molecular structures.

Commercial synthetic Fe(III)-chelate fertilizers are obtained by carrying out first the synthesis of the chelating agents and then incorporating Fe(III) from inorganic salts. The first procedures to synthesize chelating agents such as *o*,*o*EDDHA and analogues involved the use of HCN[14] and resulted in compounds of a high purity.[15] A novel method using

cyanide transfer agents instead of HCN has been developed, although it has not been scaled up for industrial applications yet.[16] The currently prevailing industrial synthesis processes[17,18] lead to products which often contain starting chemicals as well as reaction byproducts.[15,19-26] For this reason, a common characteristic of these fertilizers is the occurrence of a significant water-soluble Fe fraction not bound to the authorized chelating agents.[20-22] This is specially relevant in fertilizers containing phenolic groups in the chelating agent structure, where the Fe bound to unknown products often accounts for 40-50%.[22] The occurrence of positional isomers and poly-condensate products in Fe(III)-*o,o*EDDHA, Fe(III)-EDDHMA and Fe(III)-EDDHSA fertilizers was suggested first by HPLC/UV-Vis, and later confirmed with 1- and 2-dimensional NMR and electrospray (ESI) mass spectrometry (MS).[15,19,23,24,26] For instance, 14-30% of the water-soluble Fe content found in Fe(III)-*o,o*EDDHA fertilizers actually corresponds to Fe(III)-*o,p*EDDHA. Also, further byproducts formed in the synthesis of EDDHA were found by ESI-MS and ESI tandem MS (ESI-MS/MS).[25] This led to the acceptance of Fe(III)-*o,p*EDDHA and Fe(III)-EDDHSA condensation products by the European Legislation.[7,8] Furthermore, significant amounts of unreacted starting materials such as *p*-hydroxybenzenesulfonic (8%) and *p*-hydroxybenzoic acids (1.4-2.5%) were also found in Fe(III)-EDDHSA and Fe(III)-EDDCHA commercial fertilizers, respectively.[24]

Until now, MS studies have focused mainly on the quantification of the active ingredients of Fe(III)-chelate fertilizers using exact molecular mass[26-29] and isotopic signatures.[30] However, significant structural information can be obtained from collision-induced dissociation (CID) MS/MS, that provides molecular fragmentation patterns. This technique can be used to identify unknown molecules of the same family (i.e. Fe-containing fertilizer impurities) and also to add authority to the identification of Fe(III)-chelates in environmental matrices, which could be difficult due to the low concentrations present and the matrix complexity.[29] Several types of ESI-CID-MS^2 mass spectrometers are available, differing in the fragmentation mechanisms. For example, the fragmentation mechanism in ion trap devices is a selective excitation of the precursor ion, whereas in quadrupole time of flight (QTOF) spectrometers additional fragmentations of the product ions often occur. As far as we know, CID fragmentation patterns of synthetic Fe(III)-chelates have been only reported for Fe(III)-EDTA and Fe(III)-DTPA using a triple stage quadrupole mass spectrometer.[31]

The aim of this work was to obtain the CID fragmentation patterns of the nine major commercial synthetic Fe(III)-chelate fertilizers by HPLC/ESI-CID-MS^2, using two different mass analyzers, a spherical ion trap and a QTOF. The use of HPLC/ESI-CID-MS^2 allowed for the i) the characterization of the fragmentation patterns of Fe(III)-chelates and ii) the identification of two previously unknown, Fe-containing impurities in a Fe(III)-EDDHA commercial fertilizer.

2. EXPERIMENTAL

2.1 Chemicals and reagents

All eluents, buffers, and standard solutions were prepared with analytical grade Type I water (Milli-Q Synthesis, Millipore, Bedford, MA). Hydrochloric acid (35%) and ammonium hydroxide (25%) were purchased from Panreac Química S.A (Barcelona, Spain). Ammonium acetate (99.99%, Sigma-Aldrich, St Louis, MO), Li hydroxide

monohydrate (99.995%, Sigma-Aldrich), Na formate (99%, Sigma-Aldrich), formic acid (50%, Sigma-Aldrich), methanol and 2-propanol (both LC-MS grade, Riedel-de-Haën) were purchased from Sigma-Aldrich. Agilent tuning mix was purchased from Agilent Technologies (Waldbronn, Germany).

Chelating agents used were $Na_2H_2EDTA \cdot 2H_2O$ (99%, Merck, Barcelona, Spain), DTPA (99%, Merck), $Na_3HEEDTA$ (99%, Merck), $CDTA \cdot H_2O$ (99%, Merck), *o,o*EDDHA (98%, LCG Promochem, Barcelona, Spain), EDDHMA (98%, LCG Promochem) and *o,p*EDDHA (94.7%, provided by J.J. Lucena, Universidad Autónoma de Madrid, Spain). Fe(III)-EDDCHA and Fe(III)-EDDHSA (both 5.9% w/w Fe) were provided by J. M. García-Mina (Universidad de Navarra, Spain). Iron(III) was supplied as Fe standard Titrisol® (1 g of metal in 15% HCl, Merck, Darmstadt, Germany). Labeled ^{57}Fe oxide (Fe_2O_3, 98% Fe, 95.06% ^{57}Fe) was purchased from Cambridge Isotope Laboratories (Andover, MA).

2.2 Ferric chelate preparation

Iron(III)-chelates and ^{57}Fe labeled Fe(III)-chelates were prepared by adding slowly acidic Fe solutions (36 mM Fe and 66 mM ^{57}Fe in HCl), in 5% excess over the molar amount of chelating agent over high-pH chelating agent solutions.[32] During the addition of Fe the solution pH was maintained in the range 6-8 by adding NH_4OH. Solutions were neutralized to pH 7.0 with NH_4OH and HCl and made up to volume with Milli-Q water. Then, they were equilibrated overnight in the dark at room temperature and filtered through a 0.45 µm PVDF membrane. Stock solutions of Fe(III)-chelates (1 mM) were stored in the dark at 4 °C and standard solutions of concentrations lower than 100 µM were prepared daily from the stocks. Fe(III)-chelate standard solutions were protected from light during the preparation and until MS analysis, to avoid light-induced reduction of the Fe(III)-chelates.

2.3 Commercial fertilizers

Six commercial fertilizers, each containing at least one synthetic Fe(III)-chelate, were analyzed. Fertilizers used were commercially labeled as containing Fe(III)-EDTA (13% soluble Fe), Fe(III)-DTPA (0.3% soluble Fe), Fe(III)-HEEDTA (4.1% soluble Fe), Fe(III)-EDDHA (6% soluble Fe), Fe(III)-EDDHMA (6% soluble Fe), and Fe(III)-EDDHSA (6% soluble Fe). All soluble Fe contents indicated are those shown in the label, and are given on a w/w basis. Stock solutions (10 mM soluble Fe) were prepared by dissolving products in Milli-Q water. Solutions were filtered through a 0.45 µm PVDF membrane and stored at 4 ºC in the dark. Fertilizer solutions were protected from light during the preparation and until MS analysis, to avoid Fe(III)-chelate reduction.

2.4 ESI-MS and HPLC/ESI-MS analysis

ESI-MS analyses were carried out with three different types of mass analyzers: i) a time of flight (TOF, MicrOTOF, Bruker Daltonics, Bremen, Germany), ii) a spherical ion-trap (HCTUltra, Bruker Daltonics) and iii) a quadrupole time of flight (QTOF, MicroTof-Q, Bruker Daltonics), all of them equipped with electrospray ionization (ESI) sources. ESI-MS operating conditions were optimized by direct injection of 10 µM solutions of each Fe(III)-chelate using a syringe pump (Cole-Parmer Instrument, Vernon Hills, IL) operating at 250 $\mu L\ h^{-1}$, in order to maximize all signals and minimize in-source fragmentation and formation of adducts.

ESI-TOFMS analysis was carried out in negative ion mode, with endplate, spray tip and orifice voltages of 0.5 kV, 3.0 kV and 75 V, respectively. Nebulizer gas (N_2) pressure, drying gas (N_2) flow rate and drying gas temperature were 0.8 bar, 4.0 L min^{-1} and 180 ºC. Spectra were acquired in the 100-800 mass-to-charge ratio (*m/z*) range and the mass axis was calibrated externally using Li-formate adducts (10 mM LiOH, 0.2% (v/v) formic acid and 50% (v/v) 2-propanol).

ESI-ion-trapMS analysis was carried out in negative ion mode, with Smart Parameter Settings optimized for each *m/z*. Nebulizer (N_2) gas pressure, drying gas (N_2) flow rate and drying gas temperature were kept at 0.7 bar, 4.0 L min^{-1} and 350 ºC. Spectra were acquired in the 50-1200 *m/z* range, using the 'Ultra scan mode', and the mass axis was calibrated with tuning mix (from Agilent).

ESI-(QTOF)MS analysis was carried out in negative ion mode, with capillary and endplate offset voltages of 3.0 kV and -500 V, respectively, and collision cell energy of 10 eV. Nebulizer (N_2) gas pressure, drying gas (N_2) flow rate and drying gas temperature were 0.4 bar, 4.0 L min^{-1} and 200 ºC. Spectra were acquired in the 50-1200 *m/z* range. The mass axis was calibrated externally using Na-formate adducts (10 mM $NaHCO_2$ in 1:1 2-propanol:water).

HPLC/ESI-MS analysis was carried out with an Alliance 2795 HPLC system (Waters, Mildford, MA) coupled to the MS(TOF) or the ion-trap MS devices, and an 1100 HPLC system (Agilent) coupled to the MS(QTOF) apparatus. HPLC was carried out with a 5 µm particle size, 2.1 x 150 mm Waters Symmetry C_{18} column, protected by a 3.5 µm particle size, 2.1 x 10 mm Waters Symmetry C_{18} guard column, and a gradient of methanol and milli-Q water with a constant concentration of 1 mM ammonium acetate at pH 6.0.[28] Column temperature was 30 °C, and injection volume and flow rate were 20 µl and 100 µl min^{-1}. To allow coupling with the HPLC, nebulizer gas (N_2) pressure and drying gas (N_2) flow rate were increased to 1.6 bar and 8.0 L min^{-1} for the MS(TOF) and MS(QTOF) devices and to 2.1 bar and 8.0 L min^{-1} for the ion-trap MS apparatus.

The MS(TOF), MS(QTOF) and ion-trap systems were controlled with software packages micrOTOF Control v.2.2, micrOTOF Control v. 2.3 and Esquire Control v.6.1 (Bruker Daltonics). In all cases, HyStar v.3.2 (Bruker Daltonics) software was used to hyphenate HPLC and MS systems and Data Analysis v.4.0 (Bruker Daltonics) software was used to process data. The occurrence of Fe in any ion detected in the TOF and QTOF MS spectra was carried out automatically by applying the Isotope Cluster Analysis Chromatogram tool of the Data Analysis v.4.0 software (Bruker Daltonics). This tool also allows searching for any ion having several Fe atoms, since they have a characteristic isotopic pattern, different from that of ions with only one Fe atom. For instance, single charged ions having one or two Fe atoms were searched extracting isotope cluster analysis traces with intensity ratios of 15.7 and 7.8, respectively, using in both cases a m/z difference of 2. Subsequent manually confirmations of the Fe occurrence in ions were carried out by i) using the characteristic Fe^{56}/Fe^{54} signal intensity ratios of Fe-containing ions and ii) the comparison of the ESI-MS spectra of the ^{56}Fe(III)- and ^{57}Fe(III)-chelates, since any ion having 1, 2 or 3 Fe atoms would give *m/z* differences of 1, 2 or 3 units.

2.5 ESI-CID-MS^2 and HPLC/ESI-CID-MS^2 analysis

ESI-CID-MS^2 analyses were carried out with two different types of mass analyzers, the ion-trap and the QTOF described above, both equipped with ESI sources. MS^2 operating conditions were optimized individually in order to maximize product ion signals in both

instruments, using direct injection of 10 µM solutions of each Fe(III)-chelate. General conditions for the ion-trap and QTOF instruments were those described previously in the ESI-MS analyses section.

ESI-CID-MS^2 (ion trap) analysis was carried out in the 'Ultra scan mode' using He as collision gas, an optimal amplitude voltage of 0.5 V and an isolation width for the precursor ion of 5 *m/z* units. HPLC systems and conditions were the same as those described in the previous section. ESI-CID-MS^2(QTOF) analysis was carried out using Ar as collision gas, with an optimal collision cell energy of 15 eV and an isolation width for the precursor ion of 5 *m/z* units. Fragmentation ions were identified using the Smart Formula and Smart Formula 3D™ tools of the Data Analysis v.4.0 (Bruker Daltonics), using mass accuracy and SigmaFit™ criteria. The latter provides a numerical comparison of theoretical and measured isotopic patterns, with smaller values indicating a closer match between theoretical and measured isotopic patterns.[33,34] The occurrence of Fe in the product ions, as well as the number of Fe atoms per molecule, was determined as indicated for ESI-MS analysis (see above).

3. RESULTS

3.1 ESI-MS and HPLC/ESI-MS analysis of synthetic Fe(III)-chelates

ESI-MS analyses of Fe(III)-chelates were carried out with three different mass analyzers: TOF, QTOF and ion-trap. The ions generated and relative intensities (as % of the largest peak in each spectrum) found with the different mass analyzers are shown for each Fe(III)-chelate in Table 5-1. Since commercial EDDHSA and EDDCHA standards were not available, Fe(III)-EDDHSA and Fe(III)-EDDCHA were separated from available fertilizer mixtures by HPLC. In all cases, ion isotopic signatures indicate the presence of only 1 Fe atom per molecule. Also, isotopic signatures ruled out the possible occurrence of Fe(II)-chelates that could be formed during chelate preparation and/or electrospray ionization in negative mode; if even minor amounts of Fe(II)-chelate complexes were present, the isotopic signature will be changed, since there must be a difference of one *m/z* unit between the corresponding Fe(III)- and Fe(II)-chelate ions. Furthermore, the elemental formulae assigned to the ions indicated that the stoichiometry was always 1:1, as expected from the stoichiometry (Fe-to-chelating agent ratio) used in the preparation of Fe(III)-chelates.

Table 5-1. Ions produced in negative ESI-MS analyses of Fe(III)-chelates used as fertilizers with different mass analyzers: TOF, ion trap and QTOF. Numbers in parenthesis indicate the relative intensity signal, taking the major peak as 100%. Data were acquired by injecting 10 µM solutions of each analyte in water.

Fe(III)-chelate	Ions	TOF[a]	Ion Trap	QTOF[b]
		m/z		
Fe(III)-EDTA	$[M-H]^-$	344.0 (100)	344.0(100)	344.0(100)
	$[M-H-CO_2]^-$			300.0 (25)
	$[M-H-2CO_2]^-$			256.0 (7)
Fe(III)-DTPA	$[M-H]^-$	445.0 (100)	445.0(100)	445.0(100)
	$[M-H-CO_2H]^-$	400.0 (42)		
	$[M-H-2CO_2]^-$	357.0 (47)	357.0 (15)	357.0 (31)
	$[M-H-3CO_2]^-$			313.0 (12)
Fe(III)-HEEDTA	$[M-H]^-$	330.0 (100)	330.0(100)	330.0(100)
	$[M+Cl]^-$	365.9 (77)	365.9 (16)	365.9 (77)
	$[M-H-CO_2]^-$			286.0 (8)
Fe(III)-CDTA	$[M-H]^-$	398.0 (100)	398.0(100)	398.0(100)
	$[M-H-CO_2]^-$			354.0 (20)
	$[M-H-C_8H_{10}O_4]^-$			227.9 (8)
Fe(III)-*o,o*EDDHA	$[M-H]^-$	412.0 (100)	412.0(100)	412.0(100)
	$[M-H-CO_2]^-$			368.0 (42)
	$[M-H-2CO_2]^-$			324.0 (9)
Fe(III)-*o,p*EDDHA	$[M-H]^-$	412.0 (100)	412.0(100)	412.0(100)
	$[M+Cl]^-$	448.0 (24)	448.0 (67)	448.0 (28)
	$[M-H-CO_2]^-$			368.0 (31)
Fe(III)-EDDHMA	$[M-H]^-$	440.1 (100)	440.1(100)	440.1(100)
	$[M-H-CO_2]^-$			396.0 (20)
Fe(III)-EDDHSA	$[M-H]^-$	572.0 (100)	572.0(100)	572.0(100)
Fe(III)-EDDCHA	$[M-H]^-$	500.0 (100)	500.0(100)	500.0(100)
	$[M-H-CO_2]^-$			456.0 (13)
	$[M-H-2CO_2]^-$			412.0 (5)

[a]TOF, time of flight
[b]QTOF, quadrupole time of flight.

The most abundant ion (100% relative intensity) corresponded to the ^{56}Fe signal of the $[M-H]^-$ ions, at *m/z* values 344.0 for Fe(III)-EDTA, 445.0 for Fe(III)-DTPA, 330.0 for Fe(III)-HEEDTA, 398.0 for Fe(III)-CDTA, 412.0 for Fe(III)-*o,o*EDDHA and Fe(III)-*o,p*EDDHA, 440.1 for Fe(III)-EDDHMA, 572.0 for Fe(III)-EDDHSA and 500.0 for Fe(III)-EDDCHA. In MS(TOF) and ion trap MS analyses, the $[M-H]^-$ ion was the only one found in most cases (Table 5-1). Exceptions were Fe(III)-HEEDTA and Fe(III)-*o,p*EDDHA, where minor signals, at *m/z* 365.9 and 448.0 respectively, were assigned to chloride adducts $[M+Cl]^-$, and Fe(III)-DTPA, where a minor signal at *m/z* 357.0 was assigned to the double decarboxylation product $[M-H-2CO_2]^-$ (Table 5-1). In the latter case, an additional signal at *m/z* 400.0 in the TOFMS spectrum was assigned to a CO_2H loss. The CO_2H loss found with the Fe(III)-DTPA chelate likely occurred because among the several polyaminocarboxylate Fe(III)-chelates studied, Fe(III)-DTPA is the only one having a carboxylic group (protonated at the neutral pH used) not bound to the Fe atom.

Furthermore, when using the MS(QTOF) apparatus additional decarboxylation product ions (2-4, depending on the specific chelate) were obtained even at minimal collision energies (Table 5-1). The formation mechanism of decarboxylation product ions could be an α-cleavage, known to occur in carboxylate anions and resulting in stable carbanion species. In all Fe(III)-chelates studied, N atoms have a carboxylic group in β position, that can easily undergo decarboxylation to form the corresponding carbanion at the α position, which is stabilized by the N atom. Single decarboxylation ions were observed for all Fe(III)-chelates with the exception of Fe(III)-DTPA. The Fe-O bonds in the heptacoordinated Fe(III)-DTPA (see structure in Figure 5-1B) are weaker than those in the other Fe(III)-chelates studied, where Fe is hexa- or penta-coordinated. The CO_2 loss involving the Fe-O bond cleavage would be therefore favored in Fe(III)-DTPA and it is likely that single decarboxylated ions (which are still heptacoordinated) could not be detected due to a rapid second decarboxylation.

3.2 ESI-CID-MS^2 and HPLC/ESI-CID-MS^2 analysis of Fe(III)-chelates

ESI-CID-MS^2 and HPLC/ESI-CID-MS^2 analyses of Fe(III)- and ^{57}Fe(III)-chelates were carried out with the MS(QTOF) and ion-trap MS devices. When no standards were available, *e.g.* for the *racemic* and *meso* stereoisomers of Fe(III)-*o,o*EDDHA and Fe(III)-EDDHMA, as well as for Fe(III)-EDDCHA and Fe(III)-EDDHSA, HPLC was used to separate the chelates. In all cases, the precursor ion selected for MS^2 fragmentation was the corresponding $[M-H]^-$ ion found in the MS analyses (Table 5-1), using an isolation width of 5.0 *m/z* units (both in the case of the ion trap and the QTOF) to retain the isotopic signature of the product ions.

Fragmentation patterns of Fe(III)-chelates obtained with both mass analyzers were coherent, although differences in the number of product ions were observed. The ESI-CID-MS^2(QTOF) spectra (Figures 5-1, 5-2 and 5-4) were richer than those obtained with the ion trap (see Figure 5-S1, Supplementary Material) and showed specific differences in fragmentation patterns even in the case of Fe(III)-chelates that are positional isomers (see below). Therefore, we used subsequently Fe(III)-chelate fragmentation patterns based on the ESI-CID-MS^2 spectra obtained with the MS(QTOF) device. As an example of the quality of the data, mass accuracy and resolution of the precursor and product ions of Fe(III)-*o,o*EDDHA using MS^2(QTOF) were lower than 3.7 mDa and 0.056 FWHM (Full Width of the peak at Half of its Maximum height), respectively (Table 5-S1 in Supplementary Material).

The isotope signature of the MS^2(QTOF) product ions matched with single negatively-charged ions containing 1 Fe atom (Fe^{56}/Fe^{54} signal intensity ratio of approximately 15.69). In the case of product ions having a masked Fe isotopic signature due to the occurrence of H losses, the presence of only 1 Fe atom was confirmed using ^{57}Fe-labeled Fe(III)-chelates; all ion products of the ^{57}Fe-labeled Fe(III)-chelates differed in 1 *m/z* unit from the corresponding ^{56}Fe(III)-chelate product ions. We did not find any Fe(III)-chelate product ion having 2 or 3 ^{57}Fe atoms, that would give *m/z* differences of 2 or 3 units. Also, for all identified MS(QTOF) product ions, the most accurate elemental formulae assigned by the Data Analysis software formula tools gave an odd electronic configuration, indicating that the Fe atom was in the Fe(III) form.

At this stage, highly accurate elemental formulae were assigned for most product ions and losses with respect to the corresponding precursor ions (Figures 5-1, 5-2 and 5-4).

Based on these data, chemical structures for the product ions were proposed in some cases (Figure 5-2). According to the fragmentation pathways found, Fe(III)-chelates can be separated in two groups, non-phenolic and phenolic.

3.2.1 Non-phenolic Fe(III)-chelate CID spectra

The non-phenolic chelates Fe(III)-EDTA, Fe(III)-DTPA, Fe(III)-HEEDTA and Fe(III)-CDTA showed similar ESI-CID-MS^2(QTOF) fragmentation patterns (Figure 5-1).

The two product ions with higher *m/z* corresponded in all cases to single and double decarboxylations (at *m/z* 300.0 and 256.0 for Fe(III)-EDTA, *m/z* 286.0 and 242.0 for Fe(III)-HEEDTA, and *m/z* 354.0 and 310.1 for Fe(III)-CDTA; Figure 5-1A, 5-1C and 5-1D, respectively) except for the pentacarboxylic Fe(III)-chelate, Fe(III)-DTPA (Figure 5-1B), which gave only the double decarboxylation product (*m/z* 357.1). This could be explained (see above) based on the relative weakness of the Fe-O bonds of the heptacoordinated Fe(III)-DTPA, compared with those of the hexacoordinated Fe(III)-chelates. A triple decarboxylation product was also found in Fe(III)-EDTA (Figure 5-1A) and Fe(III)-DTPA (Figure 5-1B) at *m/z* 212.0 and 313.1, respectively. Two other product ions for Fe(III)-EDTA at *m/z* 246.0 and 228.0 corresponded to $C_4H_2O_3$ and $C_4H_4O_4$ losses, respectively (Figure 5-1A). In the case of Fe(III)-DTPA, losses of the radical $C_3H_3O_4^{\bullet}$ and the neutral fragment $C_6H_9NO_4$ yielded product ions at *m/z* 342.0 and 286.0, respectively (Figure 5-1B). Fe(III)-HEEDTA and Fe(III)-CDTA had losses of $C_4H_5NO_4$ and $C_8H_{10}O_4$, yielding product ions at *m/z* 199.0 and 228.0, respectively (Figures 5-1C and 5-1D). All these product ions were confirmed using ^{57}Fe-labelled chelate standards.

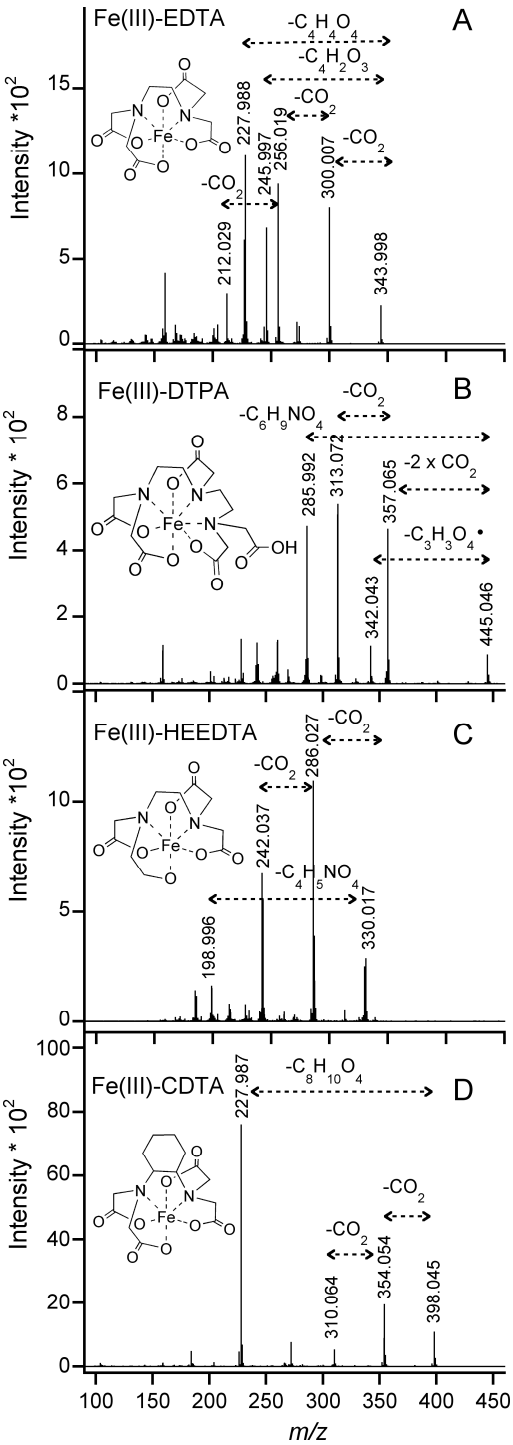

Figure 5-1. ESI-CID-MS^2 spectra of Fe(III)-EDTA (A), Fe(III)-DTPA (B), Fe(III)-HEEDTA (C) and Fe(III)-CDTA (D) obtained on the QTOF mass spectrometer in negative ion mode. The fragment losses with respect to the parent ion, and the structure of the Fe(III)-chelates are shown for each analyte. Data were acquired by injecting 10 µM solutions of each analyte in water.

3.2.2 Phenolic Fe(III)-chelate CID spectra

The CID spectra of phenolic Fe(III)-chelates are shown in Figures 5-2 and 5-4. The fragmentation of three of these chelates, Fe(III)-EDDHMA, Fe(III)-*o,o*EDDHA and Fe(III)-*o,p*EDDHA, share common characteristics; the spectra, along with a proposal for the fragmentation patterns, are shown in Figure 5-2.

The product ions generated from Fe(III)-EDDHMA (Figure 5-2A) paralleled those of Fe(III)-*o,o*EDDHA (Figure 5-2B), with a difference of 28 *m/z* units due to the two methyl groups of the aromatic rings in Fe(III)-EDDHMA. However, the positional isomer Fe(III)-*o,p*EDDHA showed product ions different from those of Fe(III)-*o,o*EDDHA, and therefore a different fragmentation pattern was proposed for this compound (Figure 5-2C). The spectra of the Fe(III)-*o,o*EDDHA and Fe(III)-EDDHMA *racemic* and *meso* stereoisomers did not show differences in fragmentation (data not shown).

In the fragmentation pattern proposed for Fe(III)-EDDHMA and Fe(III)-*o,o*EDDHA, a first decarboxylation yielded product ions at *m/z* 396.1 and 368.0, respectively (Figure 5-2A-B). A loss of the fragment $C_2H_3NO_2$ resulted in two low intensity product ions at *m/z* 367.0 and 339.0, respectively. A cleavage of the ethylendiamine group along with a loss of CO_2 resulted in the loss of $C_3H_5NO_2$ that led to the most intense product ions at *m/z* 353.0 and 325.0, respectively. Two isobaric ions not resolved by the ion-trap (see Figure 5-S1B, Supplementary Material) were resolved on the high resolution QTOF at *m/z* values of approximately 352.1 for Fe(III)-EDDHMA and 324.0 for Fe(III)-*o,o*EDDHA (Figure 5-3).

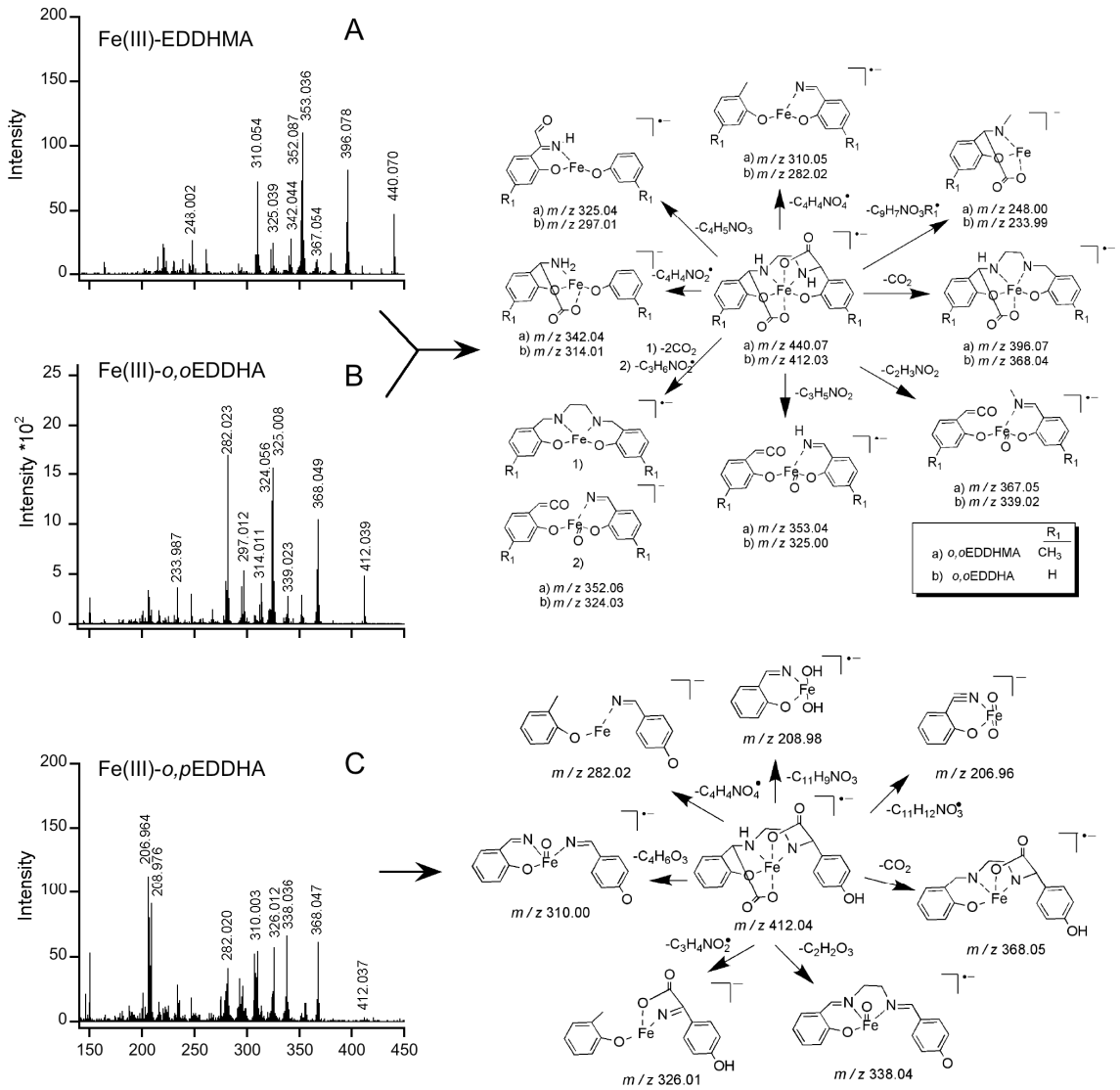

Figure 5-2. ESI-CID-MS^2 spectra of Fe(III)-EDDHMA, Fe(III)-*o,o*EDDHA and Fe(III)-*o,p*EDDHA obtained on the QTOF mass spectrometer in negative ion mode (A). Fragmentation patterns were proposed for Fe(III)-EDDHMA and Fe(III)-*o,o*EDDHA (B), and Fe(III)-*o,p*EDDHA (C). Data were acquired by injecting 10 µM solutions of each analyte in water.

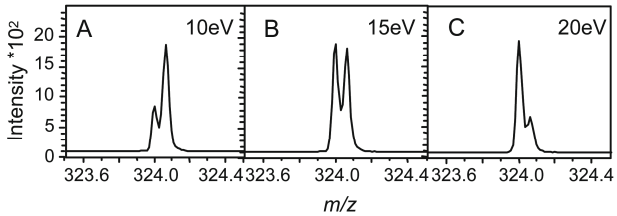

Figure 5-3. Zoom of the ESI-CID-MS^2 spectra of the two product ions of Fe(III)-*o,o*EDDHA generated at *m/z* 324.0 at three different collision energies: 10 eV (A), 15 eV (B) and 20 eV (C).

These ions had a *m/z* difference of only 0.055 and 0.057 *m/z* units, for Fe(III)-EDDHMA and Fe(III)-*o,o*EDDHA, respectively, and corresponded to a loss of $2CO_2$ for the higher *m/z* peak and a loss of $C_3H_6NO_2$ for the lower *m/z* one. The formation of these two product ions depended on collision energy, and the double decarboxylation was favored at low energies, whereas the loss of $C_3H_6NO_2$ was more pronounced at higher energies (Figure 5-3). Low intensity product ions at *m/z* 342.0 and 325.0 for Fe(III)-EDDHMA and 314.0 and 297.0 for Fe(III)-*o,o*EDDHA corresponded to losses of the radical $C_4H_4NO_2^{\bullet}$ and the neutral fragment $C_4H_5NO_3$, respectively. An intense product ion was found for both Fe(III)-EDDHMA and Fe(III)-*o,o*EDDHA at *m/z* 310.0 and 282.0, respectively, corresponding to the loss of the radical $C_4H_4NO_4^{\bullet}$. A loss of the fragment $C_9H_7NO_3R_1^{\bullet}$, which includes an aromatic ring of the chelate, accounted for product ions at *m/z* 248.0 for Fe(III)-EDDHMA and 234.0 for Fe(III)-*o,o*EDDHA. All these product ions were confirmed using ^{57}Fe-labelled chelate standards.

The Fe(III)-*o,p*EDDHA isomer had different product ions than those of the Fe(III)-*o,o*EDDHA, and therefore a different fragmentation pattern was proposed (Figure 5-2C). Two fragments were common with Fe(III)-*o,o*EDDHA, at *m/z* 368.0 and 282.0, corresponding to a single decarboxylation and the loss of the radical $C_4H_4NO_4^{\bullet}$, the latter being less intense for the *o,p* isomer than for the *o,o* one. Product ions generated by losses of $C_2H_2O_3$ and the radicals $C_3H_4NO_2^{\bullet}$ and $C_4H_6O_3$, accounted for *m/z* 338.0, 326.0 and 310.0, respectively. The most intense product ions were found at *m/z* 209.0 and 207.0, corresponding to losses of $C_{11}H_9NO_3$ and $C_{11}H_{12}NO_3^{\bullet}$, respectively. These fragmentations involved, as it also occurs with Fe(III)-*o,o*EDDHA and Fe(III)-EDDHMA, the loss of one aromatic ring, and in the case of Fe(III)-*o,p*EDDHA the intensity of these product ions were higher. All these differences in CID fragmentation between Fe(III)-*o,p*EDDHA and Fe(III)-*o,o*EDDHA were only observed on the QTOF.

The CID spectra of a second group of phenolic chelates, Fe(III)-EDDCHA and Fe(III)-EDDHSA, are shown in Figure 5-4.

A single and a double decarboxylation occurred in both cases, resulting in product ions at m/z 456.0 and 412.0 for Fe(III)-EDDCHA (Figure 5-4A) and 528.0 and 484.0 for Fe(III)-EDDHSA (Figure 5-4B). The loss of the radical $C_4H_4NO_4^{\bullet}$, that resulted in the product ion at *m/z* 370.0 for the Fe(III)-EDDCHA, was also found in other phenolic Fe(III)-chelates (Figure 5-2), but not in Fe(III)-EDDHSA (Figure 5-4B). An intense product ion, specific for Fe(III)-EDDCHA, occurs at *m/z* 250.9 and corresponds to the loss of $C_{12}H_{11}NO_5$ (Figure 5-4A). Three specific product ions for Fe(III)-EDDHSA at *m/z* 454.9, 288.9 and 200.0 corresponded to losses of $C_4H_5O_4^{\bullet}$, $C_8H_{11}O_9S^{\bullet}$ and $C_9H_8O_{12}S_2$, respectively (Figure 5-4B).

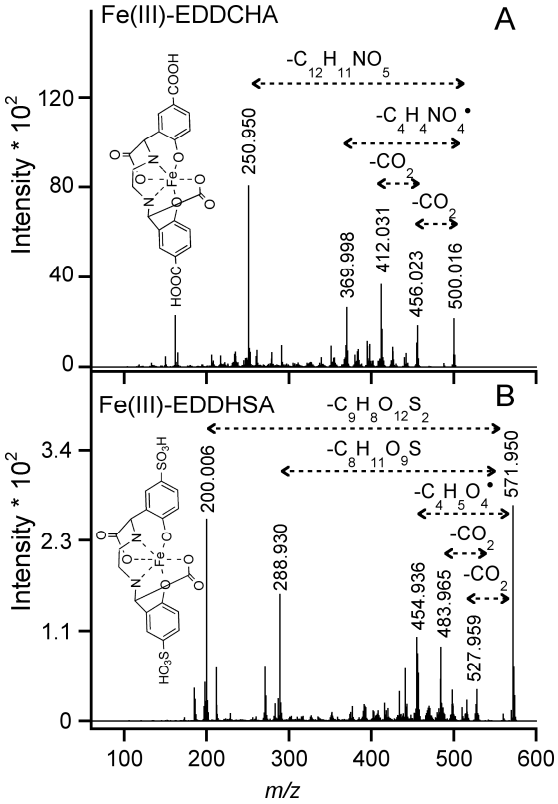

Figure 5-4. ESI-CID-MS^2 spectra of Fe(III)-EDDCHA (A) and Fe(III)-EDDHSA (B) obtained on the QTOF mass spectrometer in negative ion mode. The fragment losses with respect to the parent ion, and the structure of the Fe(III)-chelates are shown for each analyte. Data were acquired by injecting in the HPLC 50 µM solutions of each analyte in water.

3.3 Identification of unknown Fe compounds in commercial fertilizers by HPLC/ESI-MS and HPLC/ESI-CID-MS^2

Commercial fertilizers containing Fe(III)-EDTA, Fe(III)-DTPA, Fe(III)-HEEDTA, Fe(III)-EDDHA, Fe(III)-EDDHMA and Fe(III)-EDDHSA were analyzed by HPLC/ESI-CID-MS^2 with both ion trap and QTOF mass spectrometers. The precursor ion selected for CID-MS^2 fragmentation was the corresponding $[M-H]^-$ ion obtained for each Fe(III)-chelate (see Table 5-1). The CID-MS^2 spectra obtained (data not shown) always matched those generated with the Fe(III)-chelate standards.

The analysis of the Fe(III)-EDDHA fertilizer HPLC/ESI-MS data with the Isotope Cluster Analysis tool showed the occurrence of Fe-compounds different from those of Fe(III)-*o,o*EDDHA and Fe(III)-*o,p*EDDHA. These Fe compounds had *m/z* values of 453.063 and 484.020, had isotopic signatures corresponding to single charged ions and eluted as several chromatographic peaks (Figure 5-5, where panel A shows the 453.1 *m/z* trace and panel B shows the 484.0 *m/z* trace). The peak intensities of these compounds ($2x10^4$) were one order of magnitude lower than those of the main fertilizer active ingredient, Fe(III)-*o,o*EDDHA ($3x10^5$), indicating that they were relatively minor but significant impurities in the fertilizer formulation.

Using the mass exact measurements and the isotopic signatures, a list of potential elemental formulae was proposed (Table 5-2). The selection criteria for elemental formulae assignment were mass accuracy, electronic configuration, H/C ratio and SigmaFit^{TM} value. It should be noted that since synthetic Fe(III)-chelate fertilizers are prepared using inorganic Fe(III) salts, it was assumed that Fe was present as Fe(III), and therefore only an odd electronic configuration was allowed. For the ions at *m/z* 453.1, four formulae gave an error <2.25 mDa with an odd electronic configuration and a H/C ratio <2 (see Table 5-2). For the ions at *m/z* 484.0, five formulae matched the same criteria (see Table 5-2).

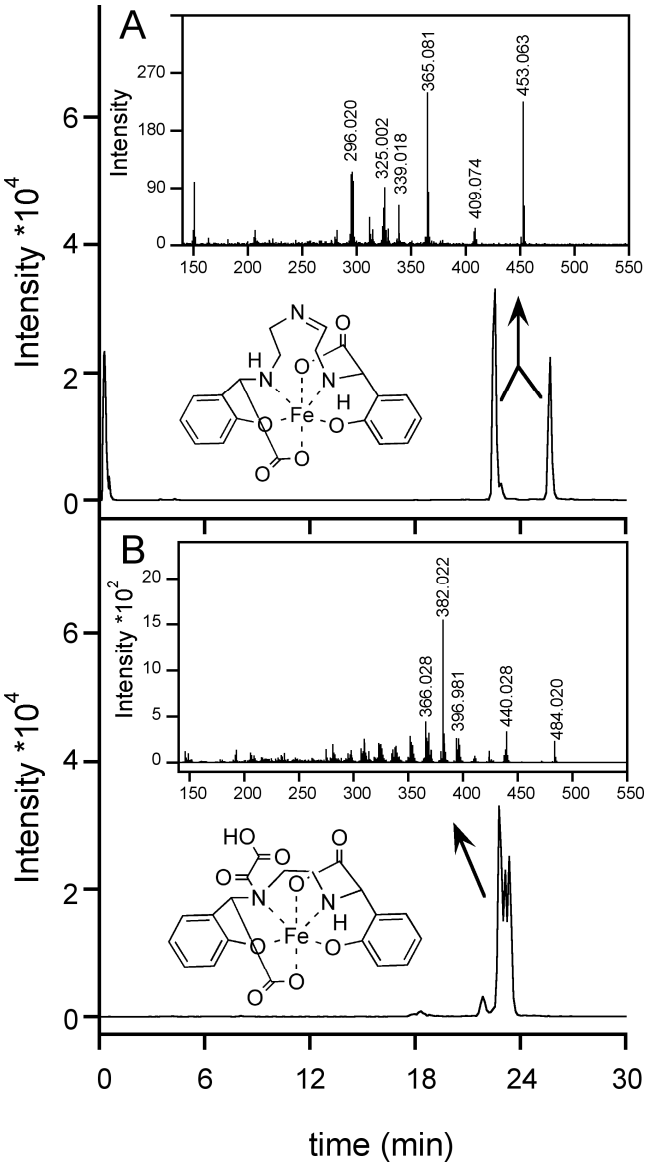

Figure 5-5. HPLC chromatograms of a commercial Fe(III)-EDDHA fertilizer solution showing peaks corresponding to Fe-containing unknowns with *m/z* 453.1 (A) and 484.0 (B). The *m/z* detection window of the HPLC-MS traces was ± 0.02 and ESI-CID-MS^2 spectra are shown in the insets. Proposals for structures of the impurities are also included. Data were acquired by injecting 41.2 mg product L^{-1} fertilizer water solutions.

Table 5-2. Potential elemental formulae for the unknown Fe-containing impurities of the Fe(III)-EDDHA-based commercial fertilizer.

Measured *m/z*	Formula	Calculated *m/z*	Mass accuracy (mDa)	Electronic configuration	H/C ratio	Sigma Fit™ value[a]
453.063	$FeC_{20}H_{19}N_3O_6$	453.062	-0.5	odd	0.9	0.032
	$FeC_{21}H_{15}N_7O_2$	453.064	0.9	odd	0.7	0.035
	$FeC_6H_{11}N_{19}O_3$	453.064	1.4	odd	1.8	0.054
	$FeC_{17}H_{11}N_{13}$	453.061	-1.8	odd	0.6	0.032
484.020	$FeC_{17}H_8N_{12}O_3$	484.019	-0.6	odd	0.4	0.020
	$FeC_{20}H_{16}N_2O_9$	484.021	0.7	odd	0.8	0.015
	$FeC_{32}H_{12}O_2$	484.019	-1.1	odd	0.3	0.064
	$FeC_{16}H_{12}N_8O_7$	484.018	-1.9	odd	0.7	0.011
	$FeC_{21}H_{12}N_6O_5$	484.022	2.1	odd	0.5	0.028

[a]SigmaFit™ is an algorithm that provides a numerical comparison of theoretical and measured isotopic patterns.

The Fe-containing ions at *m/z* 453.1 eluted as two peaks at 22.5 and 25.6 min, with identical CID-MS^2(QTOF) spectra (inset in Figure 5-5A). These retention times were very close to those of the *racemic* and *meso* isomers of Fe(III)-*o,o*EDDHA (retention times of 21.5 and 23.9 min, respectively). The CID spectra of these unknown compounds showed a similar fragmentation pattern (Figure 5-5A) to that of Fe(III)-*o,o*EDDHA (Figure 5-2B). For example, single and double decarboxylations were observed, accounting for product ions at m/z 409.1 and 365.1, respectively (Figure 5-5A inset). The two losses of CO_2 indicated that the elemental formula should have at least 4 O atoms. Among the most likely elemental formulae (Table 5-2) only $FeC_{20}H_{19}N_3O_6$ complies with this criterion, having also the best mass accuracy (-0.5 mDa error). Furthermore, the product ions at *m/z* 339.0 and 325.0, corresponding to losses of $C_4H_6N_2O_2$ and $C_5H_8N_2O_2$ respectively, were also found for Fe(III)-*o,o*EDDHA. Comparing these losses with those obtained for the Fe(III)-*o,o*EDDHA product ions having the same *m/z* ($C_2H_3NO_2$ for *m/z* 339.0 and $C_3H_5NO_2$ for *m/z* 325.0), the mass difference found was 41.03 Da in both cases, which is exactly the mass difference between the unknown compound (*m/z* 453.06) and Fe(III)-*o,o*EDDHA (*m/z* 412.03). This difference in mass corresponded, with a mass accuracy of -0.1 mDa, to a C_2H_3N fragment. Therefore, the unknown Fe-containing molecule was proposed to include a dehydrogenated diethylenetriamine instead of ethylenediamine, in a structure similar to that of Fe(III)-*o,o*EDDHA (Figure 5-5A), in good agreement with the formula $FeC_{20}H_{19}N_3O_6$ proposed above. Since that compound has 2 chiral C atoms and is asymmetric, two *d,l-racemic* mixtures are possible, and this fits with the two separate peaks found in the HPLC runs.

The Fe-containing ions at *m/z* 484.0 eluted as a group of peaks at 23 min with a common CID-MS^2(QTOF) spectrum (inset in Figure 5-5B). The retention times were also close to those of Fe(III)-*o,o*EDDHA. The CID spectra showed similar losses to those found with Fe(III)-*o,o*EDDHA (Figure 5-2B). For example, product ions at *m/z* 440.0 and 397.0 corresponded to losses of CO_2 and $C_3H_5NO_2$, respectively (Figure 5-5B), as it occurred with Fe(III)-*o,o*EDDHA at *m/z* 368.0 and 325.0. This indicated that the unknown Fe-containing molecule has a structure similar to Fe(III)-*o,o*EDDHA. The mass difference between the unknown compound (*m/z* 484.02) and Fe(III)-*o,o*EDDHA (*m/z* 412.03) was 71.99 Da, and this was assigned, with a mass accuracy of 0.3 mDa, to a glyoxyl C_2O_3

fragment. Furthermore, product ions at *m/z* 382.0 and 366.0 corresponded to losses of $C_3H_2O_4$ (C_2O_3 + COH_2) and $C_3H_2O_5$ (C_2O_3 + CO_2H_2), respectively. The $C_3H_2O_5$ loss indicated that the elemental formula should have at least 5 O atoms. Among the most likely elemental formulae (Table 5-2) only $FeC_{20}H_{16}N_2O_9$, $FeC_{16}H_{12}N_8O_7$ and $FeC_{21}H_{12}N_6O_5$ comply with this criterion, with $FeC_{20}H_{16}N_2O_9$ being the most accurate (0.7 mDa error) formula. Therefore, the unknown Fe-containing molecule is proposed to have a structure similar to that of Fe(III)-*o,o*EDDHA but including a glyoxyl group (Figure 5-5B), in good agreement with the formula $FeC_{20}H_{16}N_2O_9$. Since such compound has 3 chiral centers (2 C and 1 N), 8 isomers are possible, which could explain the elution of this compound as a group of poorly resolved peaks.

4. DISCUSSION

This is the first time, to our knowledge, that a comprehensive study on the CID spectra of the most common synthetic Fe(III)-chelates used as fertilizers has been carried out. ESI-CID-MS^2 spectra were obtained with two mass analyzers (QTOF and ion trap) that use different fragmentation mechanisms. Ferric chelate CID-MS^2(QTOF) spectra showed always a higher number of product ions than those obtained with the ion trap, and this was particularly useful to differentiate among positional isomers. The combination of rich CID spectra, high resolution and exact mass determination obtained with a QTOF analyzer was a powerful tool to characterize fragmentation patterns and identify unknown compounds in commercial Fe(III)-chelate fertilizers.

Differences in fragmentation patterns were found among synthetic Fe(III)-chelates, specially when using the QTOF analyzer. However, some common characteristics were observed, since decarboxylation fragments were always found and all product ions did contain Fe, as it could be confirmed using ^{57}Fe-labelled chelate standards. Decarboxylations were also found in the only ESI-CID-MS^2 Fe(III)-chelate study published so far (with Fe(III)-EDTA and Fe(III)-DTPA).[31] In our study, differences in Fe(III)-chelate fragmentation patterns included specific *m/z* losses and changes in the intensities of some common product ions. In most Fe(III)-chelates, major fragments corresponded to CO_2 losses. Exceptions were Fe(III)-*o,p*EDDHA, Fe(III)-EDDCHA and Fe(III)-EDDHSA, where the major product ions corresponded to losses involving one aromatic ring. This allows for an unequivocal distinction between the positional isomers Fe(III)-*o,o*EDDHA and Fe(III)-*o,p*EDDHA, which differ only in the position of an hydroxyl group, resulting in hexa- and penta-Fe coordination environments, respectively (Figure 5-2). For these isomers, specific fragments were also found, *e.g.* at *m/z* 310.0 for the *o,p* isomer and 314.0 for the *o,o*, which could permit direct determination, without a previous chromatographic separation, using selected reaction monitoring MS^2 analysis (*e.g.* 412.0 → 310.0 for the *o,p* isomer and 412.0 → 314.0 for the *o,o* one). This could be particularly interesting in fertilizer quality analyses, since both isomers are generally present in commercial Fe(III)-EDDHA products, with the *o,o* and *o,p* isomers accounting for 75-85% and 25-15%, respectively, of the total Fe(III)-EDDHA.[22,28] Unfortunately, a real simultaneous quantification of the Fe(III)-EDDHA positional isomers was not possible in our study, since we had only a very small quantity of the product *o,p*EDDHA (not commercially available) kindly provided by Professor J. J. Lucena (Autonomous University of Madrid, Spain). In the case of the stereoisomers *racemic* and *meso* Fe(III)-*o,o*EDDHA, spectra obtained with both mass analyzers were indistinguishable, and a previous separation (*e.g.* by HPLC) is still necessary.

Two previously unknown, Fe-containing compounds have been found in a Fe(III)-EDDHA-based commercial fertilizer. The structure of these compounds was elucidated using the HPLC-ESI-CID-MS^2(QTOF) data. They have molecular formulae $FeC_{20}H_{20}N_3O_6$ (M.W. 454.1 g/mol) and $FeC_{20}H_{17}N_2O_9$ (M.W. 485.0 g/mol), and show retention times and fragmentation patterns similar to those of Fe(III)-*o,o*EDDHA ($FeC_{18}H_{17}N_2O_6$ and M.W. 413.0 g/mol). The unknown compound with M.W. 454.1 was proposed to be similar to Fe(III)-*o,o*EDDHA but including a dehydrogenated diethylenetriamine instead of ethylenediamine; this compound was putatively assigned to Fe(III)-N-(2-aminoethylidene)-1,2-diaminoethane-N',N"-bis(*o*-hydroxyphenylacetate) and was present as two *d,l-racemic* mixtures that were well separated by HPLC (Figure 5-5A). The presence of such compound in Fe(III)-EDDHA fertilizers can be explained by the fact that the starting reagent ethylenediamine[18] may contain significant amounts of diethylenetriamine.[35] Also, dehydrogenation of diethylenetriamine has been reported in a Pt(IV)-chelate.[36] The unknown compound with M.W. 485.0 was proposed to be Fe(III)-*o,o*EDDHA with an additional glyoxyl group; this compound was putatively assigned to Fe(III)-ethylenediamine-N-glyoxyl-N,N'-bis(*o*-hydroxyphenylacetate) (Figure 5-5B). The eight possible stereoisomers of this compound were not well separated by HPLC (Figure 5-5B). The presence of this type of molecule in Fe(III)-EDDHA fertilizers can be explained by the fact that the starting reagent glyoxylic acid[18] may contain oxalic acid,[37] that may react with one of the amine groups of ethylenediamine, forming a glyoxyl group. Until now, only EDDHA positional isomers (*o,p* and *p,p*-EDDHA)[19] and poly-condensate compounds derived from the chelating agent synthesis have been reported as byproducts occurring in Fe(III)-EDDHA commercial fertilizers.[23,25] No poly-condensate compounds were found in the present study, probably because they are not eluted in the chromatographic conditions used. All these impurities may supply Fe to plants to some extent, and for instance Fe(III)-*o,p*EDDHA was found to be at least as effective as Fe(III)-*o,o*EDDHA in nutrient solutions[38,39] but not in calcareous soils,[40] although a mixture of Fe(III)-EDDHA poly-condensate byproducts was not effective to correct Fe-deficiency.[25]

This study opens the possibility for applying HPLC/ESI-CID-MS^2(QTOF) analyses to identify Fe-containing byproducts in commercial synthetic Fe(III)-chelate fertilizers. The most common approaches to identify impurities in fertilizers until now are HPLC/UV/Vis[15,19] or HPLC/ESI-MS[26] direct analyses and NMR analyses of deferrated fertilizers.[23,24] Since the HPLC/UV-Vis and HPLC/ESI-MS direct analyses provide limited structural information compared to NMR, the identity of the impurities has been putatively assigned in most cases, with only a few confirmations through the synthesis of standards (*e.g.* Fe(III)-*o,p*EDDHA). Although NMR provides comprehensive structural information allowing for the direct identification of the deferrated impurities, it is not possible to know whether they bound Fe in the fertilizer. Also, the deferration process could alter the original composition of the fertilizer, as it occurs with Fe-EDDHA fertilizers, where significant losses of *o,o*EDDHA and its minor isomers were observed upon deferration.[41] A more recent approach to investigate the impurities of Fe-chelate fertilizers (applied to Fe(III)-EDDHA) was based on modifying the industrial chelating agent synthesis to favor the generation of byproducts, and then analyzing the reaction mixtures by ESI-CID-MS^2.[25] However, the presence of most of the impurities found in that study has not been confirmed so far in commercial fertilizers.

In summary, the HPLC-ESI-CID-MS^2(QTOF) technique allows for differentiating among the most common synthetic polyaminocarboxylate Fe(III)-chelates used as fertilizers. This study is not intended to replace the existing methods for quantification of the synthetic Fe(III)-chelates in fertilizers, but adds authority to their identification,

specially in complex matrices where isobaric interferences can appear. Furthermore, the methodology has permitted the identification of two Fe-containing impurities in a Fe(III)-EDDHA-based commercial fertilizer. Therefore, the data found and the methodology described here shall provide a basis to further characterize the Fe soluble fraction of Fe(III)-chelate fertilizers, which would be of importance in issues related to crop Fe fertilization, both from agricultural (fertilizer quality and efficiency, etc.) and environmental (persistence, mobility, metal mobilization, etc.) points of view.

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SUPPLEMENTARY MATERIAL

Figure 5-S1. ESI-CID-MS^2 spectra of synthetic polyaminocarboxylate Fe(III)-chelates used as fertilizers obtained on the spherical ion-trap mass spectrometer.

Table 5-S1. ESI-CID-MS^2(QTOF) mass accuracy and resolution data for the precursor and product ions of Fe(III)-*o,o*EDDHA.

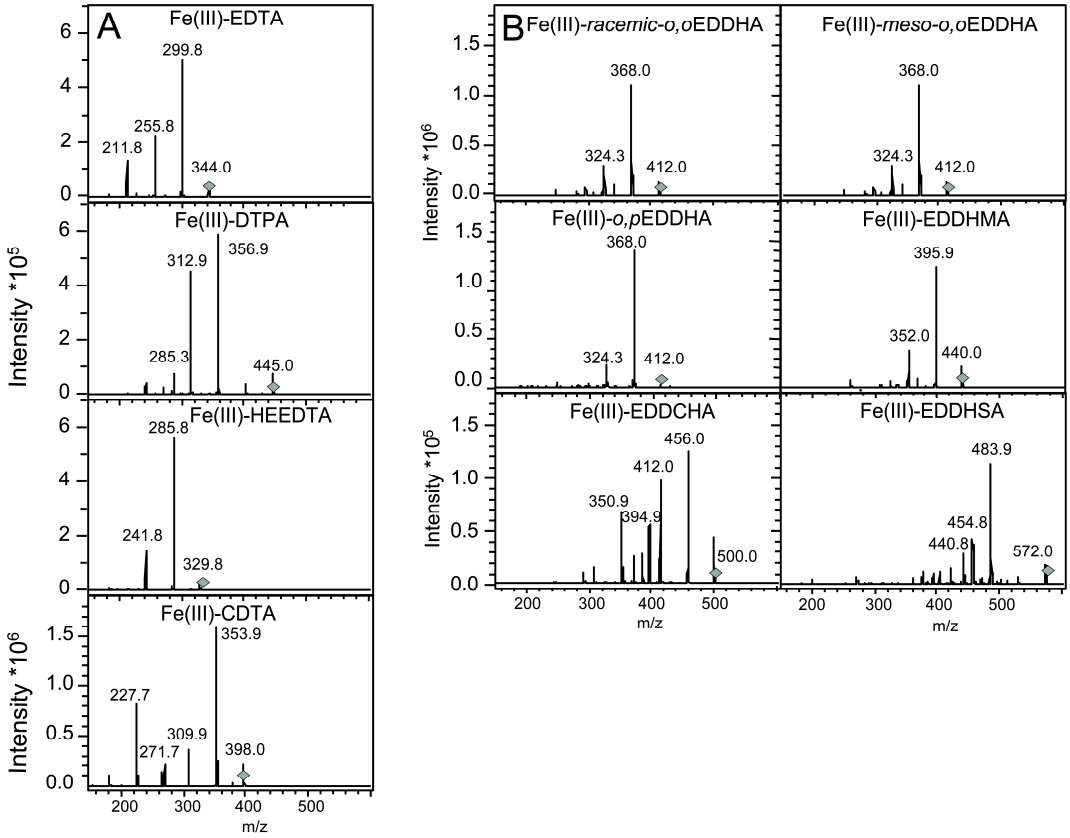

Figure 5-S1. ESI-CID-MS^2 spectra of non-phenolic (A: Fe(III)-EDTA, Fe(III)-DTPA, Fe(III)-HEEDTA and Fe(III)-CDTA) and phenolic (B: Fe(III)-*racemic-o,o*EDDHA, Fe(III)-*meso-o,o*EDDHA, Fe(III)-*o,p*EDDHA, Fe(III)-EDDHMA, Fe(III)-EDDCHA and Fe(III)-EDDHSA) Fe(III)-chelates obtained on the spherical ion-trap mass spectrometer in negative ion mode. Data were acquired by injecting 10 μM solutions of each analyte in water.

Table 5-S1. ESI-CID-MS^2(QTOF) data for the precursor and product ions of Fe(III)-*o,o*EDDHA: measured mass to charge (*m/z*) ratio, formula assigned to the measured *m/z*, calculated *m/z*, error (in mDa), resolution (FWHM[a]) and SigmaFit[b] value for the precursor (in bold) and product ions. Data were analyzed with the Smart Formula™ tool of the Data Analysis software.

m/z measured	Formula	*m/z* calculated	Error (mDa)	Resolution (FWHM[a])	SigmaFit™ value[b]
		QTOF			
412.039	**$FeC_{18}H_{16}N_2O_6$**	**412.036**	**-2.9**	**0.0516**	**0.105**
368.049	$FeC_{17}H_{16}N_2O_4$	368.047	-2.1	0.0481	0.168
325.008	$FeC_{15}H_{11}NO_4$	325.004	-3.7	0.0450	0.044
324.056	$FeC_{16}H_{16}N_2O_2$	324.057	0.8	0.0509	0.096
323.999	$FeC_{15}H_{10}NO_4$	323.997	-2.6	0.0556	0.406
314.011	$FeC_{14}H_{12}NO_4$	314.012	0.7	0.0380	0.085
297.012	$FeC_{14}H_{11}NO_3$	297.009	-2.6	0.0419	0.085
282.023	$FeC_{14}H_{12}NO_2$	282.022	-0.7	0.0376	0.085
233.987	$FeC_9H_8NO_3$	233.986	-0.9	0.0347	0.062

[a]FWHM: Full Width of the peak at Half of its Maximum height.

[b]SigmaFit™ is an algorithm that provides a numerical comparison of theoretical and measured isotopic patterns.

Capítulo 6

METALLOMICS

Enviado

USING A DUAL-STABLE ISOTOPE TRACER METHOD TO STUDY THE UPTAKE, XYLEM TRANSPORT AND DISTRIBUTION OF Fe AND ITS CHELATING AGENT FROM STEREOISOMERS OF A XENOBIOTIC Fe(III)-CHELATE USED AS FERTILIZER IN Fe-DEFICIENT STRATEGY I PLANTS

Irene Orera[a], José A. Rodríguez-Castrillón[b], Mariella Moldovan[b], José I. García-Alonso[b], Anunciación Abadía[a], Javier Abadía[a] and Ana Álvarez-Fernández[a]*

[a]Department of Plant Nutrition, Aula Dei Experimental Station (CSIC), P.O. Box 13034, 50080 Zaragoza, Spain.

[b]Department of Physical and Analytical Chemistry, University of Oviedo, 33006 Oviedo, Spain.

USING A DUAL-STABLE ISOTOPE TRACER METHOD TO STUDY THE UPTAKE, XYLEM TRANSPORT AND DISTRIBUTION OF Fe AND ITS CHELATING AGENT FROM STEREOISOMERS OF A XENOBIOTIC Fe(III)-CHELATE USED AS FERTILIZER IN Fe-DEFICIENT STRATEGY I PLANTS

Summary: A dual-stable isotope tracer experiment was carried out with Fe-deficient sugar beet plants grown hydroponically and resupplied with differentially Fe labeled *racemic* and *meso* Fe(III)-chelates of the ethylendiamine di(*o*-hydroxyphenylacetic) acid (*o,o*EDDHA). No short-term Fe isotope exchange reactions occurred in the nutrient solution and plants did not discriminate between ^{54}Fe and ^{57}Fe. After 3-6 h, stable Fe isotopes, chelating agents and chelates were analyzed in roots, xylem sap and leaves by ICP-MS and HPLC-ESI/TOFMS. Ferric chelate reductase rates, xylem transport and total uptake were 2-fold higher with the *meso* isomer than with the *racemic* one. Both chelating agent isomers were incorporated and distributed by plants at similar rates, in amounts one order of magnitude lower than those of Fe. After 6 h of Fe resupply, most of the Fe acquired was localized in roots, whereas most of the chelating agent was in leaves. In a separate experiment, Fe-deficient sugar beet and tomato plants were treated with different concentrations of Fe(III)-*o,o*EDDHA (with a *meso*/*racemic* ratio of 1). The xylem sap Fe concentration at 24 h was unaffected by the chelate concentration, with xylem Fe(III)-*o,o*EDDHA accounting for 1-18% of total Fe and xylem *meso*/*racemic* ratio close to 1. Although most of the Fe coming from Fe(III)-*o,o*EDDHA was taken up through a reductive dissociative mechanism, a small part of the Fe may be taken up *via* non-dissociative mechanisms.

Keywords: Chelate · Inductively coupled mass spectrometry · EDDHA · Electrospray mass spectrometry · Iron.

1. INTRODUCTION

Ethylendiamine di(*o*-hydroxyphenylacetic) acid (*o,o*EDDHA) is a xenobiotic water-soluble compound, known for its high Fe(III)-binding ability.[1] In biomedical studies, Fe(III)-*o,o*EDDHA has been used in magnetic resonance imaging[2] and positron emission tomography.[3] In agricultural practice, the deficiency of Fe in crops (also called Fe-chlorosis) is commonly remedied by soil applications of Fe(III)-*o,o*EDDHA.[4] Iron chlorosis is characterized by a marked decrease in leaf chlorophyll, that in turn results in losses in crop quality and yield[5]; the most common cause of Fe deficiency is low soil Fe bioavailability, due to the occurrence of this metal in oxy-hydroxide insoluble forms. The efficiency of Fe(III)-*o,o*EDDHA as an Fe fertilizer is due to the remarkable stability over a wide range of pH values[1] and the low reactivity in soils.[6] In spite of the high cost of Fe(III)-*o,o*EDDHA, fertilization with this compound is one of the most widespread practices to control Fe-chlorosis in high-value crops and soil-less horticulture.[4]

Iron uptake from this and other Fe(III)-chelates is carried out in the so-called Strategy I plant species by a root ferric chelate reductase (FCR) enzyme.[7,8] Radioactivity assays using ^{59}Fe(III)-^{14}C-EDDHA in the nutrient solution confirmed that Fe-deficient Strategy I plants have a splitting Fe uptake mechanism, with final root ^{59}Fe/^{14}C ratios being very different in Fe-deficient and Fe-sufficient plants (approximately 25 and 6, respectively)[9]. In the case of Fe-deficient plants most of the EDDHA remains in the soil solution, possibly facilitating further solubilization and transport of native Fe to the rhizosphere, in what has been called 'shuttle effect'.[4,10]

In spite of the existence of the splitting uptake mechanism, both synthetic Fe(III) chelating agents and Fe(III)-chelates have been found in plants after fertilization. Radioactivity assays with ^{14}C labeled Fe(III)-EDDHA found significant amounts of ^{14}C in Strategy I and II plants,[9,11,12] indicating that either the whole chelating agent or one or more breakdown product(s) could enter the plant. Iron(III)-EDDHA was also found, using UV-Vis detection, in xylem exudates of zinnia, sunflower and soybean[13], as well as in tobacco[14], pepper, lettuce and tomato tissues.[15] Recently, significant amounts of both Fe(III)-*o,o*EDDHA and *o,o*EDDHA have been found in sugar beet and tomato tissues using a very selective and sensitive technique, HPLC-ESI/TOFMS.[16]

Commercially available Fe(III)-*o,o*EDDHA fertilizers contain a mixture (approximately 1:1) of two groups of stereoisomers, the *meso* form [(R,S-Fe(III)-*o,o*EDDHA)] and the *d,l racemic* mixture [(R,R-Fe(III)-*o,o*EDDHA) + (S,S-Fe(III)-*o,o*EDDHA)]. Although most studies on Fe(III)-*o,o*EDDHA have not considered the existence of this heterogeneity, significant differences in the physico-chemical properties of the stereoisomers have been observed, including different stability constants of the complexes with Fe (log K values of 35.86 and 34.15 for the *racemic* and *meso* forms, respectively)[1] and different chromatographic mobilities.[17] Moreover, some animal and plant studies have reported differences in the *in vivo* behavior of the stereoisomers. *Meso* Fe(III)-*o,o*EDDHA was found to bind non-specifically to the bilirrubin binding site of human serum albumin, whereas the *racemic* isomer used a single high-affinity site.[18] An *in vivo* study with rats reported a more rapid blood clearance and a higher Fe liver uptake in the case of the *meso* stereoisomer as compared to the *racemic* one.[19] In Strategy I plant species grown in a nutrient solution containing both isomers (in a 1:1 ratio), it was found that *meso* Fe(III)-*o,o*EDDHA was preferentially depleted from the solution.[20,21] However, this effect did not occur with Strategy II species lacking FCR activity.[20] Although bean plants treated separately with *racemic* and *meso* ^{59}Fe(III)-EDDHA did not show significant differences in

Fe plant uptake,[22] higher *in vivo* root FCR reduction rates were found for the *meso* than for the *racemic* isomer in cucumber plant.[23]

A new approach to study plant Fe uptake and translocation is the use of multiple-stable isotope tracer methodologies, which also provide the possibility to carry out long-term experiments (Fe has four stable isotopes: ^{54}Fe, ^{56}Fe, ^{57}Fe and ^{58}Fe; see review by Álvarez-Fernández)[24]. The main drawbacks of using stable isotopes are the high cost and the special instrumentation (e.g. inductively coupled plasma mass spectrometry (ICP-MS) or electrospray time-of-flight mass spectrometry (ESI/TOFMS)) needed to determine them in complex matrices. ICP-MS has been used successfully for Fe stable isotope studies in plants because of the high selectivity and sensitivity; Fe uptake from ^{57}Fe(III)-*o,o*EDDHA and ^{57}Fe(III)-*o,p*EDDHA has been recently studied in cucumber[25], tomato and peach[26] plants. A different technique, HPLC-ESI/TOFMS, has been recently used for the determination of stable Fe isotope-labeled synthetic Fe(III)-chelates in agricultural matrices[27] and plant tissues[16]. Iron-57 Mössbauer spectroscopy has also been used to study the chemistry of Fe in plants[28], although this technique has sensitivity limitations.

No data are yet available, to the best of our knowledge, on the possible direct competition during plant uptake between the two Fe(III)-*o,o*EDDHA stereoisomers. The aim of this work was to study the uptake, movement and distribution of Fe and ligands from *racemic* and *meso* Fe(III)-*o,o*EDDHA chelates, when applied simultaneously to the roots of Fe-deficient Strategy I plants. Sugar beet plants grown in nutrient solution were treated with a 1:1 mixture of *racemic* and *meso-o,o*EDDHA isomers, each one labeled with a different Fe stable isotope (^{54}Fe or ^{57}Fe). Then, the contents of the Fe stable isotopes in different plant parts were determined by ICP-MS, whereas those of the ligands (*racemic* and *meso-o,o*EDDHA) were determined by HPLC-ESI/TOFMS.

2. EXPERIMENTAL

2.1 Chemicals and reagents

All solutions were prepared with analytical grade type I water (Milli-Q Synthesis, Millipore, Bedford, MA) with the exception of nutrient solutions, which were prepared with analytical grade type II water (Milli-RX20, Millipore). Reagent-grade glacial acetic acid, hydrochloric acid (35%) and ammonium hydroxide (25%) were purchased from Panreac Química S.A (Barcelona, Spain). Ammonium acetate (99.99%, Sigma), Li hydroxide monohydrate (99.99%, Sigma), nitric acid (65% TraceSELECT Ultra, Fluka), formic acid (50%, Sigma), methanol and 2-propanol (both LC-MS grade, Riedel-de-Haën) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Nitric acid 68% and H_2O_2 30% (Suprapur) were obtained from Merck (Darmstadt, Germany).

Pure chelating agents used were ethylenediamine tetraacetic acid (EDTA; 99%, Merck), *o,o*EDDHA (98%, a 1:1 mixture of *racemic* and *meso*; LCG Promochem, Barcelona, Spain) and ethylenediamine-N-N'bis(2-hydroxy-4-methylphenylacetic) acid (*o,o*EDDHMA; 98%, LCG Promochem). A commercial Fe(III)-EDDHA (6% soluble Fe) was also used in some experiments (Syngenta Agro, Madrid, Spain). Natural abundance Fe (thereafter called Fe^{nat}, with isotopic abundances of 5.8% ^{54}Fe, 91.7% ^{56}Fe, 2.1% ^{57}Fe and 0.2% ^{58}Fe) was supplied as Fe standard Titrisol (1000 mg Fe in 15% HCl, Merck). Labeled ^{57}Fe oxide (Fe_2O_3, 98% Fe, 95.06% ^{57}Fe) and labeled ^{54}Fe oxide (Fe_2O_3, 99.8% Fe, 99.8% ^{54}Fe) were obtained from

Cambridge Isotope Laboratories (Andover, MA). A certified Fe standard solution for ICP-MS analyses (Iron ICP standard, 1000 mg L^{-1}) was purchased from Merck.

2.2 Preparation of Fe(III)-*o,o*EDDHA chelates

Racemic- and *meso*-*o,o*EDDHA were separated from 98% *o,o*EDDHA by selective precipitation of *racemic*-*o,o*EDDHA with Mg(II), using the method of Yunta *et al*.[1] The purity of *racemic*- (99.7%) and *meso*-*o,o*EDDHA (95.0%) was determined by HPLC-ESI/TOFMS (see below; Orera *et al*.)[16]. Stock solutions (1 mM) of *racemic* Fe^{nat}(III)-*o,o*EDDHA, *racemic* ^{54}Fe(III)-*o,o*EDDHA, *racemic* ^{57}Fe(III)-*o,o*EDDHA, *meso* Fe^{nat}(III)-*o,o*EDDHA, *meso* ^{54}Fe(III)-*o,o*EDDHA, *meso* ^{57}Fe(III)-*o,o*EDDHA, Fe^{nat}(III)-*o,o*EDDHA, Fe^{nat}(III)-EDTA and Fe^{nat}(III)-*o,o*EDDHMA were prepared by slowly adding acidic Fe solutions (9.2 mM ^{54}Fe, 8.2 mM ^{57}Fe or 35.8 mM Fe^{nat} in 15-35% HCl, in 5% excess over the molar amount of each chelating agent) over high-pH pure chelating agent solutions.[17] During the addition of Fe, pH was maintained in the range 6-8 by adding NH_4OH. Solutions were set to pH 7.0 with NH_4OH and HCl and made up to volume with Milli-Q water, equilibrated overnight in the dark at room temperature and filtered through a 0.45 µm PVDF membrane. Standard solutions of Fe(III)-chelates with concentrations lower than 100 mM were prepared daily from stocks. Solutions of a Fe(III)-EDDHA commercial fertilizer (6% of soluble Fe) were used where indicated.

2.3 Plant material

Sugar beet (*Beta vulgaris* L. cv. 'Orbis') and tomato (*Lycopersicon esculentum* L. cv. 'Tres Cantos') plants were grown in a growth chamber with a photosynthetic photon flux density (PPFD) of 350 µmol m^{-2} s^{-1} photosynthetically active radiation at leaf level, a photoperiod of 16 h light/8 h dark, a temperature of 23/18 ºC day/night, and 80% relative humidity. Seeds were germinated and grown in vermiculite for two weeks. Seedlings were grown for two more weeks in half-strength Hoagland solution with 45 µM Fe^{nat}(III)-EDTA, pH 5.5, and then transplanted to plastic buckets (four plants per bucket) containing half-strength Hoagland solution with 0 µM Fe. pH was buffered at approximately 7.0 by adding 1 mM NaOH and 1 g l^{-1} $CaCO_3$ for sugar beet plants and 0.5 mM KOH for tomato plants. The total amount of solution was held constant by refilling with distilled water. Sugar beet and tomato plants showing Fe chlorosis were used after growth in 0 µM Fe for approximately 14 and 10 d, respectively.

Some Fe-deficient sugar beet and tomato plants were transferred to two L plastic pots (four plants per pot) containing nutrient solution supplemented with different concentrations of a Fe(III)-EDDHA commercial fertilizer solution (0, 35, 73, 107, 262, 427, 693 and 1470 µM for sugar beet and 0, 31, 54, 82, 177, 383, 798 and 2038 µM for tomato). Plants were treated with Fe for 24 h. Xylem sap was collected from sugar beet and tomato plants by centrifugation of petioles[29] and plant de-topping[30], respectively, filtered through 0.45 µm PVDF membranes and stored at -20 ºC until analysis. All fresh xylem sap samples were assessed for contamination using c-mdh (EC 1.1.1.37) as a cytosolic marker[29], and no contamination was found.

In a different experiment, Fe-deficient sugar beet plants (70±19 g FW per plant) were transferred to two L plastic pots (four plants per pot) containing nutrient solution supplemented with two different Fe treatments: (A) 30 µM *racemic* ^{54}Fe-*o,o*EDDHA plus 30 µM *meso* ^{57}Fe-*o,o*EDDHA or (B) 30 µM *racemic* ^{57}Fe-*o,o*EDDHA plus 30 µM *meso*

^{54}Fe-*o,o*EDDHA (see Fig. 6-1 for a description of the protocol). Three and six h after Fe resupply, xylem sap, young and old leaves and main and fine roots were collected from Fe-resupplied and untreated Fe-deficient plants grown in the same conditions. Leaves and roots were washed with distilled water. Approximately one g of fresh material was frozen in liquid N_2 and stored at -20 ºC. The rest of leaves and roots were dried in an oven at 60 ºC, ground in a ZrO_2 ball mill (MM301, Retsch, Haan, Germany) and stored at room temperature until analysis.

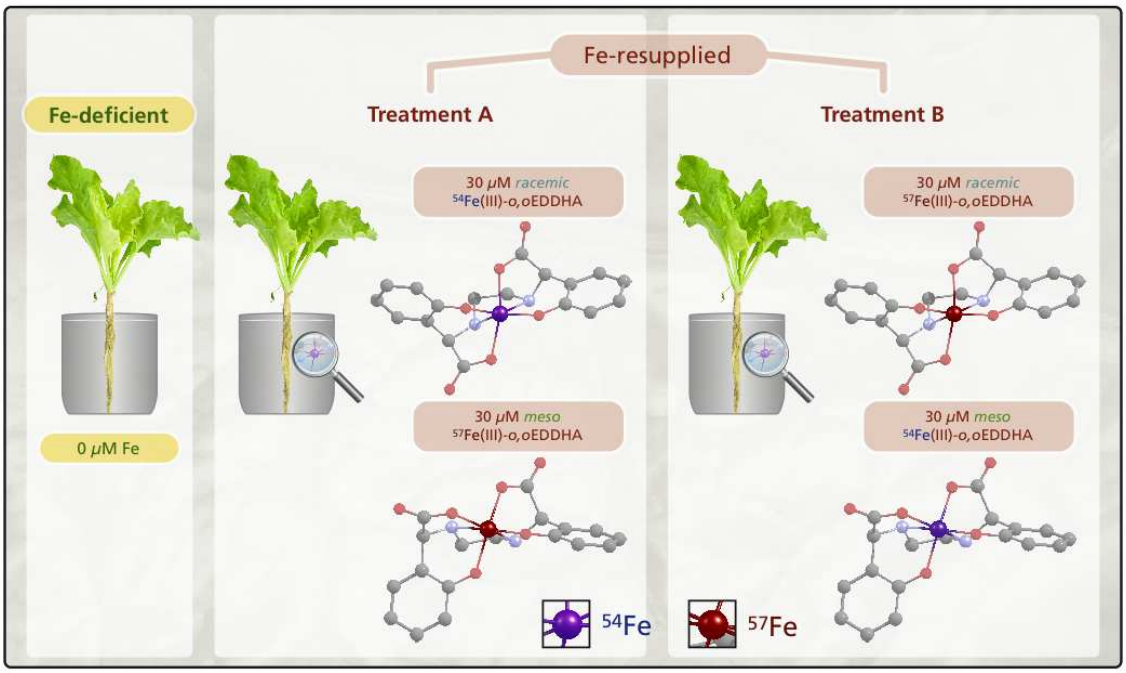

Figure 6-1. Protocol for the dual-stable Fe isotope tracer experiment. Iron deficient sugar beet plants were treated with differentially labeled (^{57}Fe and ^{54}Fe) *meso* and *racemic* Fe(III)-*o,o*EDDHA.

2.4 Root Fe(III)-chelate reductase activity

The FCR activity of roots of intact, illuminated Fe-deficient sugar beet plants was followed by measuring spectrophotometrically the formation of the Fe(II) complex with 4,7-diphenyl-1,10-phenanthrolinedisulfonic acid (BPDS) at 535 nm.[23,31] Individual plants were placed into 0.5 L beakers containing 1 mM MES (pH 6.0), 100 µM BPDS and 60 µM *racemic* or *meso* Fe^{nat}(III)-*o,o*EDDHA in MilliQ water. Beakers were fully covered with aluminum foi1 to exclude light, the solution was aerated continuously and aliquots (3 mL) were removed at 0.5, 1, 2 and 5 h. Since both Fe(III)-chelates used as substrates had a significant absorption at 535 nm, measurements were also made at 480 nm to calculate both the Fe(III)$BPDS_3$ and Fe(III)-chelate concentrations in the FCR assay solutions.[23] Beakers without plants were used to correct for non-enzymatic Fe(III) reduction.

2.5 Extraction of *o,o*EDDHA from plant materials

Sugar beet tissue extraction was performed as described in Orera *et al.*[16] Frozen leaves (1 g FW) or roots (2 g FW) were ground with 2 mL ammonium acetate (1 mM pH 6.0) containing the internal standard Fe^{nat}(III)-*o,o*EDDHMA in a ZrO_2 ball mill at 30 rps for 1-2 min. The suspension was centrifuged at 12000 *g* for 20 min at 4 ºC and the supernatant was collected. The pellet was resuspended with 1 mL of ammonium acetate solution and centrifuged again. This step was repeated once more and supernatants were combined and made up to 5 g final weight with ammonium acetate solution. An aliquot of the extract (2 g) was treated with NH_4OH until basic pH was attained, an excess of Fe(III) was added and pH was adjusted to 6-7 with HCl. Solutions were filtered and made up to 5 g final weight.

2.6 Plant tissue digestion

Xylem sap was digested with 1% HNO_3 (TraceSELECT Ultra). Tissues were digested in a closed vessel microwave oven (Milestone Model MLS 1200, Sorisole, Italy). Approximately 0.1 g or 0.05 g of sugar beet leaves or roots, respectively, were digested with 6 mL HNO_3 (26%) and 2 mL H_2O_2 (30%). The microwave digestion program was 3 min at 95 ºC, 10 min at 160 ºC, 3 min at 185 ºC and 15 min at 185 ºC. At the end of the process, digests were diluted to 50 mL with Milli-Q water and stored at 4º C until analysis.

2.7 Iron determination

Total Fe was determined in sugar beet and tomato xylem sap by graphite furnace atomic absorption spectrometry (Varian 3000, Palo Alto, CA, USA). The isotope composition of Fe (^{54}Fe, ^{56}Fe, ^{57}Fe and ^{58}Fe) in sugar beet leaves, roots and xylem sap was determined by quadrupole ICP-MS. The device (model Agilent 7500ce; Agilent Technologies, Tokyo, Japan) was equipped with an octapole collision cell to remove polyatomic interferences and was operated with a RF power of 1500 W and cooling and carrier gas flows of 15 and 1.1 L min^{-1}, respectively. The collision cell was operated with an He gas flow of 4.0 mL min^{-1} and a cell exit, octapole and quadrupole bias voltages of -72.0, -18.0 and -16.0 V, respectively. Torch position and ion lens voltage settings were optimized daily for optimum sensitivity with a solution of 1 ng g^{-1} Li, Co, Y, Tl and Ce mixture in 1% (w/w) HNO_3. A solution of 1% (w/w) HNO_3 was also used to check the background level caused by polyatomic Ar interferences. The possible contribution of isobaric interferences of $^{40}Ca^{16}O$, $^{40}Ca^{16}O^{1}H$, ^{54}Cr and ^{58}Ni was corrected mathematically by measuring ion signals at masses 43 for Ca, 52 for Cr and 60 for Ni. Mass bias correction was carried out internally by minimizing the sum of squared residuals.[32] The ICP-MS instrument was controlled with ICP-MS ChemStation software version B.03.04 (Agilent Technologies).

The isotope composition of the stable isotope-enriched Fe spikes was determined by direct ICP-MS nebulization. Measured isotope abundances (in % ^{54}Fe, ^{56}Fe, ^{57}Fe and ^{58}Fe) were 0.14, 4.74, 94.63 and 0.49 for the ^{57}Fe-enriched solution and 96.32, 3.26, 0.41 and 0.01 for the ^{54}Fe-enriched solution, respectively. Iron enriched solutions were mixed with the Fe^{nat} certified standard and Fe concentrations were calculated by reverse isotope dilution analysis.

The determination of the molar fractions of ^{54}Fe, ^{57}Fe and Fe^{nat} present in the different plant materials was carried out by direct ICP-MS nebulization of the plant digests after 1:10 dilution and employing a modification of the mathematical isotope pattern deconvolution

procedure described elsewhere.[25] For the purpose of this study three different isotope patterns were fitted to the linear model as shown in equation [1]:

$$\begin{bmatrix} A_s^{54} \\ A_s^{56} \\ A_s^{57} \\ A_s^{58} \end{bmatrix} = \begin{bmatrix} A_{54}^{54} & A_{57}^{54} & A_{nat}^{54} \\ A_{54}^{56} & A_{57}^{56} & A_{nat}^{56} \\ A_{54}^{57} & A_{57}^{57} & A_{nat}^{57} \\ A_{54}^{58} & A_{57}^{58} & A_{nat}^{58} \end{bmatrix} \begin{bmatrix} x_{54} \\ x_{57} \\ x_{nat} \end{bmatrix} + \begin{bmatrix} e^{54} \\ e^{56} \\ e^{57} \\ e^{58} \end{bmatrix} \qquad [1]$$

, where A_s, A_{54}, A_{57} and A_{nat} correspond to the measured (A_s, A_{54} and A_{57}) and theoretical (A_{nat}) isotope abundances for the different isotopes of Fe in the samples (s) and the different tracer solutions (^{54}Fe and ^{57}Fe enriched spikes). The molar fractions x_{54}, x_{57} and x_{nat} in the samples are then obtained by multiple linear regression where e is the error vector. In this case, the extra degree of freedom is used for internal mass bias correction.[25] A second ICP-MS measurement was carried out with plant digests to determine the total ^{54}Fe, ^{57}Fe and Fe^{nat} concentrations by isotope dilution analysis, using the ^{54}Fe-enriched solution as a spike.

2.8 *Racemic* and *meso* Fe(III)-*o,o*EDDHA determination in nutrient solution and xylem sap

Racemic and *meso* Fe(III)-*o,o*EDDHA were determined directly by HPLC-ESI/TOFMS in nutrient solutions and in sugar beet and tomato xylem sap.[16,27]

2.9 *Racemic*- and *meso-o,o*EDDHA determination in plant tissues

In sugar beet tissue (leaf and root) extracts, *racemic*- and *meso-o,o*EDDHA were determined by HPLC-ESI/TOFMS as *racemic* and *meso* Fe(III)-*o,o*EDDHA, after addition of an excess of Fe(III) to ensure the chelation of all *o,o*EDDHA chemical forms with Fe(III).[16] This is a necessary step, because the chelating agent *o,o*EDDHA could occur in tissue homogenates in chemical forms other than Fe(III)-*o,o*EDDHA.[16]

2.10 Statistical analysis

ANOVA tests were carried out using the SPSS software v. 15.0 (SPSS Inc.). Means were compared using the Duncan´s LSD test.

3. RESULTS

3.1 Effect of Fe(III)-*o,o*EDDHA nutrient solution concentration on the xylem sap concentrations of Fe and *racemic* and *meso* Fe(III)-*o,o*EDDHA

Sugar beet and tomato Fe-deficient plants were treated with different concentrations of commercial Fe(III)-*o,o*EDDHA, which contains a ca. 1:1 mixture of *racemic* and *meso* isomers. Xylem sap concentrations of total Fe and *racemic* and *meso* Fe(III)-*o,o*EDDHA were measured 24 h after treatment (Fig. 6-2). Iron(III)-*o,o*EDDHA treatments increased the xylem sap total Fe concentrations in sugar beet and tomato (Fig. 6-2a and b, respectively). However, all different Fe(III)-*o,o*EDDHA treatments led to similar (not significantly different at $p<0.05$) Fe concentrations in the xylem sap of each species, with values being 4- to 5-fold larger in tomato than in sugar beet.

Significant amounts of the Fe(III)-*o,o*EDDHA chelate were found in sugar beet and tomato xylem sap in all the Fe(III)-*o,o*EDDHA treatments (Fig. 6-2c and d). In the 30-450 µM Fe(III)-*o,o*EDDHA concentration treatment range, the xylem sap Fe(III)-*o,o*EDDHA concentration was approximately 0.3 and 1.5 µM in sugar beet and tomato, respectively. However, treatments with higher Fe(III)-*o,o*EDDHA concentrations (700-800 µM) led to 6- to 7-fold increases in Fe concentrations in both species. The highest Fe(III)-*o,o*EDDHA concentrations used did not change significantly the xylem sap Fe(III)-*o,o*EDDHA concentration in sugar beet (Fig. 6-2c), whereas in tomato plants a further increase was found (Fig. 6-2d).

The *meso*/*racemic* ratio in xylem sap was approximately 1.0 in all Fe(III)-*o,o*EDDHA treatments and in both plant species. Visual symptoms (red leaf spots) of Fe(III)-*o,o*EDDHA toxicity were observed only in tomato plants treated with the highest chelate concentration. Iron(III)-*o,o*EDDHA was 1-8% and 1-18% of the total Fe in the xylem sap of sugar beet and tomato plants, respectively, depending on the Fe(III)-*o,o*EDDHA concentrations applied; the highest values were found in plants treated with the highest Fe(III)-*o,o*EDDHA concentrations.

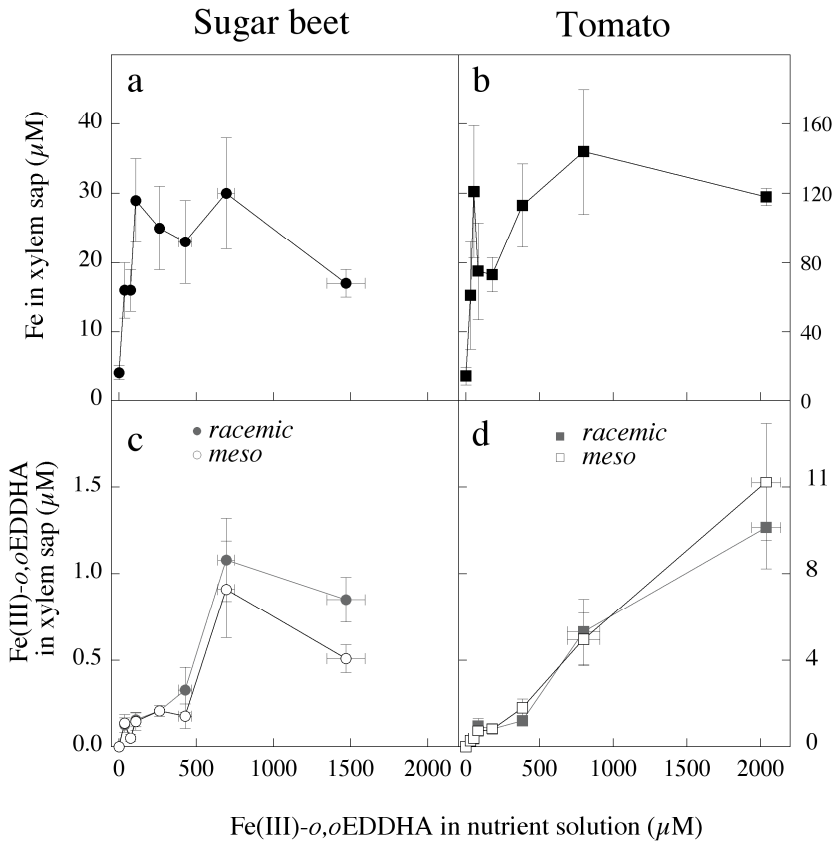

Figure 6-2 Iron (**a**, **b**) and *racemic* and *meso* Fe(III)-*o,o*EDDHA (**c, d**) xylem sap concentrations (in µM) in Fe-deficient sugar beet (**a, c**) and tomato (**b, d**) plants, after 24 h of Fe resupply with different Fe(III)-*o,o*EDDHA concentrations (0, 35, 73, 107, 262, 427, 693 and 1470 µM for sugar beet and 0, 31, 54, 82, 177, 383, 798 and 2038 µM for tomato). Data are means ± SE (n = 4-10).

3.2 Plant uptake, long-distance transport and distribution of Fe and *racemic*- and *meso*-*o,o*EDDHA

We applied simultaneously two Fe(III)-*o,o*EDDHA stereoisomers, each one labeled with a different stable Fe isotope, to Fe-deficient sugar beet plants for 3 and 6 h. Treatments were: (A) 30 µM *racemic* ^{54}Fe(III)-*o,o*EDDHA plus 30 µM *meso* ^{57}Fe(III)-*o,o*EDDHA, and (B) 30 µM *racemic* ^{57}Fe(III)-*o,o*EDDHA plus 30 µM *meso* ^{54}Fe(III)-*o,o*EDDHA (see Fig. 6-1). First, to assess the possible existence of isotope exchange reactions, the concentrations of Fe(III)-*o,o*EDDHA isomers in the nutrient solutions were determined by HPLC-ESI/TOFMS at 0 and 6 h after preparation, measuring ^{54}Fe, ^{56}Fe, ^{57}Fe and ^{58}Fe by ICP-MS and isotope dilution analysis. *Racemic* and *meso* Fe(III)-*o,o*EDDHA were well separated (Fig. 6-3). Also, the high mass resolution of the detector allowed for the accurate determination of the *racemic* and *meso* Fe(III)-*o,o*EDDHA chelates with ^{54}Fe (Fig. 6-3a and b), ^{56}Fe (present in a very small amount, Fig. 6-3c and d) and ^{57}Fe (Fig. 6-3e and f); chromatograms for the *o,o*EDDHA chelates with ^{54}Fe, ^{56}Fe and ^{57}Fe were extracted at *m/z* ratios 410.0, 412.0 and 413.0, respectively, with a precision of ±0.02 *m/z*. In both treatments, the relative amounts of the different stable Fe isotope-labeled Fe(III)-

*o,o*EDDHA chelates in nutrient solutions were similar 0 and 6 h after preparation: values found were (in %; ^{54}Fe-*o,o*EDDHA : ^{56}Fe-*o,o*EDDHA : ^{57}Fe-*o,o*EDDHA) 92 : 5 : 3 for the *racemic* and 1 : 4 : 95 for the *meso* isomer in treatment A and 3 : 6 : 91 for the *racemic* and 96 : 1 : 3 for the *meso* in treatment B. These results indicate that metal isotope exchange reactions between isomers did not occur in the nutrient solution in the time period considered.

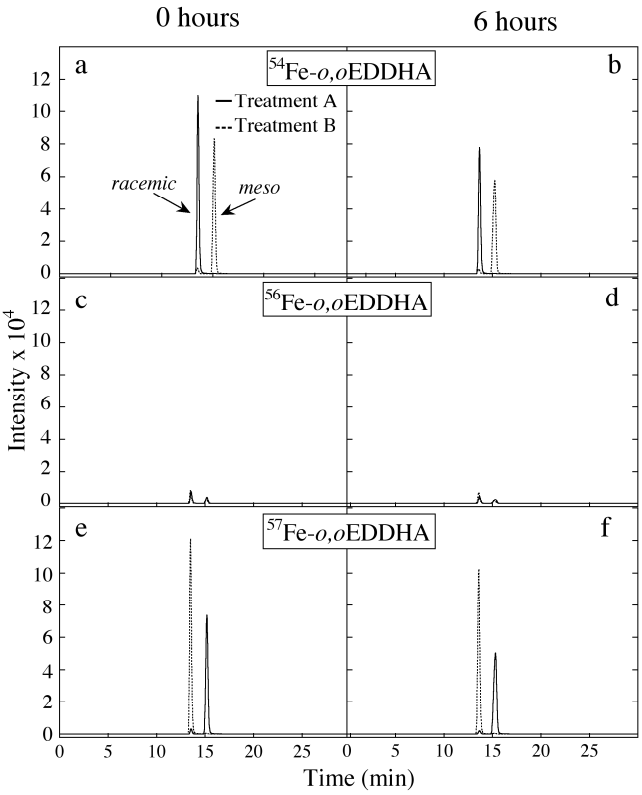

Figure 6-3. Chromatograms of nutrient solutions in treatments (A) and (B) at 0 and 6 h after preparation, showing the molecular traces of ^{54}Fe(III)-*o,o*EDDHA (*m/z* 410.0; **a**, **b**), ^{56}Fe(III)-*o,o*EDDHA (*m/z* 412.0; **c**, **d**) and ^{57}Fe(III)-*o,o*EDDHA (*m/z* 413.0; **e**, **f**).

3.2.1 Iron and *racemic*- and *meso-o,o*EDDHA uptake and allocation in sugar beet plants

The amounts of Fe taken up by sugar beet plants after resupply with 30 µM *racemic* plus 30 µM *meso* Fe(III)-*o,o*EDDHA, labeled differentially with ^{54}Fe and ^{57}Fe in treatments A and B, were determined at 3 and 6 h as indicated above. In both treatments, the amounts of Fe coming from the *racemic* and *meso* Fe(III)-*o,o*EDDHA complexes were calculated from the concentrations of the corresponding Fe stable isotope, and they are thereafter called $Fe^{ex\text{-}rac}$ and $Fe^{ex\text{-}meso}$, respectively. The amount of Fe pre-existing in roots (Fe^{nat}) was calculated as indicated in Rodríguez-Castrillón *et al.*[25] Results show that plants did not discriminate between ^{54}Fe and ^{57}Fe: both Fe-resupply treatments (A and B) led to similar values, both in terms of Fe ($Fe^{ex\text{-}rac}$ and $Fe^{ex\text{-}meso}$) and chelating agents (*racemic*- and *meso-o,o*EDDHA) (values not significantly different at $p<0.05$; not shown). Therefore, data

presented in the next paragraphs are always the average of 8 replicates, 4 from Treatment A and 4 from Treatment B.

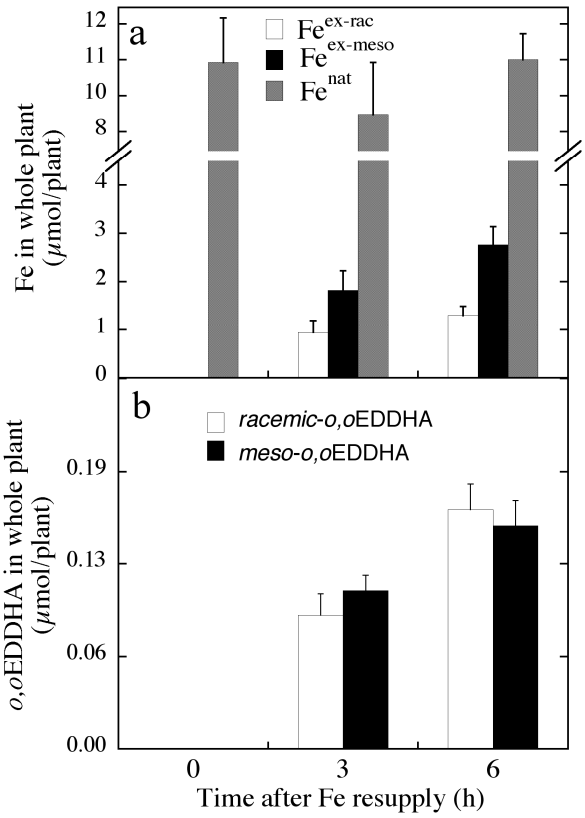

Figure 6-4. Iron (**a**) and *racemic*- and *meso-o,o*EDDHA (**b**) contents (in µmol plant^{-1}) in whole sugar beet plants at 0, 3 and 6 h after Fe-resupply. Data include Fe tracers ($Fe^{ex\text{-}rac}$ and $Fe^{ex\text{-}meso}$) coming from nutrient solution Fe(III)-*o,o*EDDHA and native Fe preexisting before Fe resupply (Fe^{nat}). Data are means ± SE (n = 8).

The amount of Fe taken up from the solution by whole sugar beet plants ($Fe^{ex\text{-}meso}$ + $Fe^{ex\text{-}rac}$) was only a small fraction of the total Fe (Fe^{nat} + $Fe^{ex\text{-}meso}$ + $Fe^{ex\text{-}rac}$), both at 3 and 6 h after Fe-resupply. The amounts of Fe^{nat} in the plant did not change significantly upon Fe resupply (Fig. 6-4a). The amounts of $Fe^{ex\text{-}meso}$ taken up from the nutrient solution were higher than those of $Fe^{ex\text{-}rac}$ at both sampling times (Fig. 6-4a). At 6 h, increases in $Fe^{ex\text{-}meso}$ and $Fe^{ex\text{-}rac}$ were found as compared to the values observed at 3 h (1.5- and 1.4-fold, respectively; Fig. 6-4a). The $Fe^{ex\text{-}meso}/Fe^{ex\text{-}rac}$ ratios in whole sugar beet plants were approximately 2.3 and 2.2 at 3 and 6 h, respectively (Table 6-1), values significantly different (at $p<0.001$) to the Fe^{meso}/Fe^{rac} ratio of 1.0-1.1 found in nutrient solutions. This indicates that plants took up Fe preferentially from the *meso* isomer as compared to the *racemic* one.

Table 6-1 Ratios of $Fe^{ex\text{-}meso}/Fe^{ex\text{-}rac}$, *meso*-/*racemic*- *o,o*EDDHA and and *meso*/*racemic* Fe(III)-*o,o*EDDHA found in nutrient solutions, whole plants and in different plant parts 3 and 6 h after Fe resupply. Data are means ± SE (n = 8). Different letters mean significant differences ($p < 0.05$) between values in the same column. n.s. means not significant differences.

	3h	6h
Material	$Fe^{ex\text{-}meso}/Fe^{ex\text{-}rac}$	
Nutrient solution	1.1 ± 0.1^{a}	1.0 ± 0.0^{a}
Whole plant	2.3 ± 0.3^{bc}	2.2 ± 0.2^{b}
Fine roots	3.0 ± 0.6^{c}	2.4 ± 0.3^{b}
Main root	2.4 ± 0.4^{bc}	1.8 ± 0.2^{b}
Xylem sap	1.9 ± 0.2^{b}	2.1 ± 0.2^{b}
Old leaves	1.6 ± 0.1^{b}	2.1 ± 0.2^{b}
Young leaves	1.5 ± 0.1^{b}	1.9 ± 0.1^{b}
meso/*racemic* Fe(III)-*o,o*EDDHA		
Nutrient solution	$1.1 \pm 0.1^{n.s.}$	$1.0 \pm 0.1^{n.s.}$
Xylem sap	$0.9 \pm 0.1^{n.s.}$	$1.3 \pm 0.2^{n.s.}$
meso/*racemic*-*o,o*EDDHA		
Whole plant	$1.2 \pm 0.1^{n.s.}$	$1.0 \pm 0.1^{n.s.}$
Fine roots	$1.3 \pm 0.3^{n.s.}$	$1.4 \pm 0.2^{n.s.}$
Old leaves	$1.3 \pm 0.1^{n.s.}$	$1.0 \pm 0.1^{n.s.}$
Young leaves	$1.0 \pm 0.1^{n.s.}$	$0.9 \pm 0.1^{n.s.}$

The contents of $Fe^{ex\text{-}rac}$, $Fe^{ex\text{-}meso}$ and Fe^{nat} were determined in four different sugar beet tissues: fine and main roots, old leaves and young leaves (Fig. 6-5). Pre-existing Fe (Fe^{nat}) was the main component of total Fe in all plant parts, with contents being unaffected by Fe resupply (Fig. 6-5). Young leaves, old leaves, main root and fine roots accounted for 12-17, 22-25, 3-4 and 58-59% of the total Fe taken up from the nutrient solution at 3-6 h (Fig. 6-5); values increased significantly in young leaves (2-fold) and fine roots (1.4-fold) from 3 to 6 h after Fe resupply. The $Fe^{ex\text{-}meso}/Fe^{ex\text{-}rac}$ ratios were in the range 1.5–3.0 in all tissues (Table 6-1), values significantly higher (at $p<0.01$) than the Fe^{meso}/Fe^{rac} ratio of 1.0-1.1 found in nutrient solutions. The highest $Fe^{ex\text{-}meso}/Fe^{ex\text{-}rac}$ ratios were found in fine roots, with values of 3.0 and 2.4 at 3 and 6 h, respectively (Table 6-1).

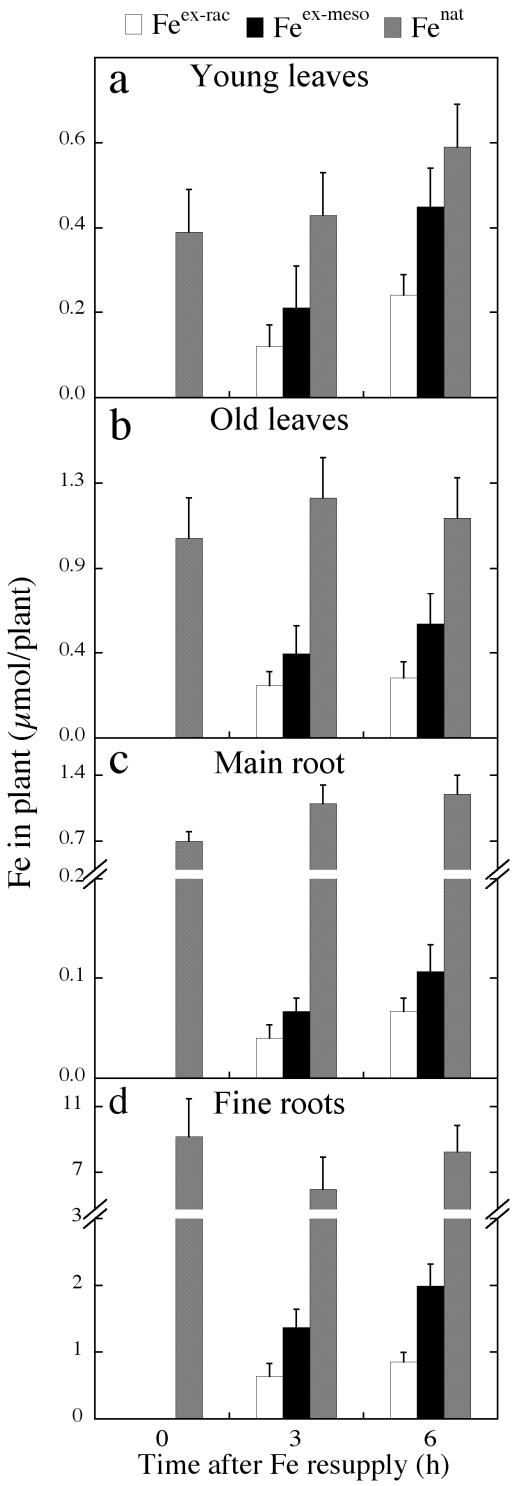

Figure 6-5. Contents (in µmol plant-1) of Fe tracers ($Fe^{ex\text{-}rac}$ and $Fe^{ex\text{-}meso}$) and native Fe preexisting before Fe resupply (Fe^{nat}) in young leaves, old leaves, main and fine roots of sugar beet plants at 0, 3 and 6 h after Fe-resupply. Data are means ± SE (n = 8).

When considering the total chelating agents (estimated after adding an excess of Fe(III), see Orera *et al.*),[16] the contents of *meso*- and *racemic-o,o*EDDHA in the whole plant were similar, both at 3 and 6 h after Fe resupply (Fig. 6-4b). At 6 h, the contents of *meso*- and *racemic-o,o*EDDHA increased (1.5- and 1.7-fold, respectively) when compared to the values found at 3 h (Fig. 6-4b). Ratios of *meso-o,o*EDDHA/*racemic-o,o*EDDHA in sugar beet plants were approximately 1.2 and 1.0 at 3 and 6 h, respectively (Table 6-1), indicating that the *racemic* and *meso* isomers were incorporated at similar rates. *Racemic*- and *meso-o,o*EDDHA were similarly distributed in all tissues (Fig. 6-6), with *meso/racemic* ratios in the range 0.9–1.3 (Table 6-1); main roots were not analyzed because the analyte recoveries were not good enough for this tissue (data not shown). Most of the *o,o*EDDHA was located in leaves (78-84%), with values of approximately 98±18 and 10±12 nmol plant^{-1} in young and old leaves, respectively; although there was a tendency to increase with time, no significant differences between treatment times were found.

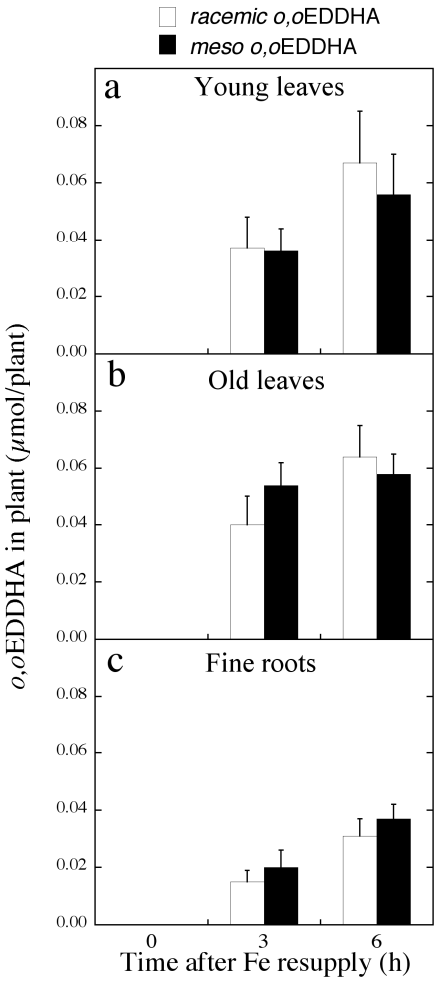

Figure 6-6. Contents (in µmol plant^{-1}) of *racemic*- and *meso-o,o*EDDHA in young leaves, old leaves and fine roots of sugar beet plants at 0, 3 and 6 h after Fe-resupply. Data are means ± SE (n = 8).

The amounts of *meso*- and *racemic-o,o*EDDHA taken up by the plant were one order of magnitude lower than those of Fe, with plant $Fe^{ex\text{-}meso}$/*meso-o,o*EDDHA, $Fe^{ex\text{-}rac}$/*racemic-o,o*EDDHA and ($Fe^{ex\text{-}meso}$ + $Fe^{ex\text{-}rac}$)/(*racemic*- + *meso-o,o*EDDHA) ratios in the ranges 17-

18, 8-10 and 13-14, respectively. The total amount of *o,o*EDDHA (*racemic* plus *meso*) found would account, if totally chelated with Fe, for 8-7% of the total $Fe^{ex\text{-}meso}$ + $Fe^{ex\text{-}rac}$, respectively, at 3-6 h after resupply. The contents of *racemic*- and *meso-o,o*EDDHA found in young and old leaves and fine roots were 1-2 orders of magnitude lower than those of Fe. The $Fe^{ex\text{-}meso}$/*meso-o,o*EDDHA ratios were in the ranges of 8-6, 8-10 and 45-57 in young leaves, old leaves, and fine roots, respectively, whereas $Fe^{ex\text{-}rac}$/*racemic-o,o*EDDHA ratios were in the ranges of 3-4, 5-6 and 23-35 in the same plant tissues. The total amount of *o,o*EDDHA (*racemic* plus *meso*) found would account, if totally chelated with Fe, for 22-25, 14-14 and 2-3% of the total $Fe^{ex\text{-}meso}$ + $Fe^{ex\text{-}rac}$ in young leaves, old leaves and fine roots, respectively, at 3-6 h after resupply.

3.2.2 Concentrations of Fe and *racemic* and *meso* Fe(III)-*o,o*EDDHA in sugar beet xylem sap

Iron in the xylem sap could came from three different Fe sources: i) Fe (^{54}Fe or ^{57}Fe) taken up from the nutrient solution *racemic* Fe(III)-*o,o*EDDHA isomer ($Fe^{ex\text{-}rac}$), ii) Fe (^{54}Fe or ^{57}Fe) taken up from the nutrient solution *meso* Fe(III)-*o,o*EDDHA isomer ($Fe^{ex\text{-}meso}$), and iii) Fe^{nat} pools pre-existing in roots. Results show again that plants did not discriminate between ^{54}Fe and ^{57}Fe: the two Fe-resupply treatments, A and B, led to similar values (not significantly different at $p<0.05$; not shown), both for xylem Fe and for xylem *racemic* and *meso* Fe(III)-*o,o*EDDHA. Therefore, data presented in the next paragraphs are the average of 8 replicates, 4 from Treatment A and 4 from Treatment B.

The xylem concentrations of Fe^{nat} did not change significantly upon Fe resupply (Fig. 6-7a). The concentrations of $Fe^{ex\text{-}rac}$ and $Fe^{ex\text{-}meso}$ transported in xylem sap were in the ranges 0.4-0.9 and 0.7-1.9 µM, respectively, with values 2.1- and 2.7-fold higher at 6 than at 3 h for $Fe^{ex\text{-}rac}$ and $Fe^{ex\text{-}meso}$, respectively (Fig. 6-7a). The xylem sap $Fe^{ex\text{-}meso}/Fe^{ex\text{-}rac}$ ratios were 1.9 and 2.1 at 3 and 6 h, respectively (Table 6-1), values significantly different (at $p<0.01$) from the 1.0-1.1 Fe^{meso}/Fe^{rac} ratio found in nutrient solutions. This indicates that the xylem was relatively enriched in $Fe^{ex\text{-}meso}$ when compared to the nutrient solution.

When considering the chelates, the concentrations of *racemic* and *meso* Fe(III)-*o,o*EDDHA in xylem sap were measurable but low, with the two isomers being transported in similar amounts (*meso*/*racemic* ratios of 0.9 and 1.3 at 3 and 6 h, respectively (Table 6-1). Total Fe(III)-*o,o*EDDHA concentrations being transported at 3 and 6 h were 0.10 and 0.06 µM, respectively (values not significantly different at $p<0.05$; Fig. 6-7b). The relative amounts of Fe(III)-chelate transported in the xylem were two orders of magnitude lower than those of Fe, with the concentration of *meso* + *racemic* Fe(III)-*o,o*EDDHA accounting for only 1 and <1% of the total Fe at 3 and 6 h, respectively. When looking only at the labeled Fe coming from the nutrient solution, the concentration of *meso* + *racemic* Fe(III)-*o,o*EDDHA accounted for 10 and 2% of the ($Fe^{ex\text{-}meso}$ + $Fe^{ex\text{-}rac}$) at 3 and 6 h, respectively.

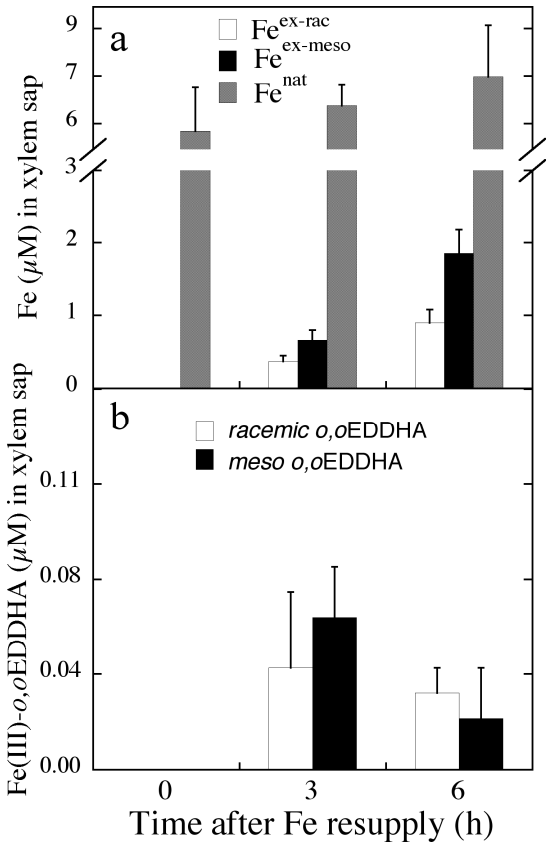

Figure 6-7. Iron (**a**) and *racemic* and *meso*-*o,o*EDDHA (**b**) concentrations (in µM) in Fe-deficient sugar beet xylem sap at 0, 3 and 6 h after Fe-resupply. Data include Fe coming from nutrient solution (*racemic* and *meso* Fe(III)-*o,o*EDDHA) and native Fe before Fe-resupply (Fe^{nat}). Data are means ± SE (n = 8).

3.2.3 Iron reduction from *racemic* and *meso* Fe(III)-*o,o*EDDHA substrates

Root FCR activity was measured with intact Fe-deficient sugar beet plants at different times, using as reduction substrates *racemic* and *meso* Fe^{nat}(III)-*o,o*EDDHA separately (Fig. 6-8). In both cases the amount of Fe reduced was linear with time, confirming previous results with sugar beet.[33] The root FCR activity was 2.7-fold higher with the *meso* isomer than with the *racemic* one.

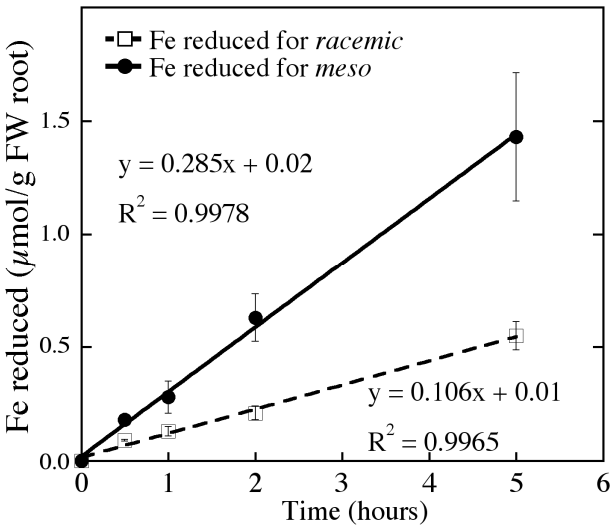

Figure 6-8. Plots of Fe(II) reduced (in µmol Fe g^{-1} FW root) versus time obtained using illuminated whole Fe-deficient sugar beet plants, 60 µM *racemic* or *meso* Fe(III)-*o,o*EDDHA and 100 µM BPDS. Data are means ± SE (n = 4).

4. DISCUSSION

When Fe-deficient *Beta vulgaris* plants (a Strategy I species) were treated with a 1:1 mixture of *racemic* and *meso* Fe(III)-*o,o*EDDHA labeled differentially with ^{54}Fe and ^{57}Fe, Fe was preferentially taken up from the *meso* isomer when compared to the *racemic* one (ratios $Fe^{ex\text{-}meso}/Fe^{ex\text{-}rac}$ of 2.2). This is the first time that a dual-stable Fe isotope tracer approach has been used in plant Fe nutrition. Plants did not discriminate between ^{54}Fe and ^{57}Fe and no short-term Fe isotope exchange reactions occurred with the Fe(III)-chelates used. The preferential plant uptake found for the $Fe^{ex\text{-}meso}$ is in agreement with the relative depletion of the *meso* Fe(III)-*o,o*EDDHA isomer observed previously in nutrient solutions with other Strategy I plant species.[20,21] Previous studies comparing the efficiency of the *racemic* and *meso* Fe(III)-*o,o*EDDHA have always applied these isomers individually, not as a mixture; these studies gave contradictory results, since bean plants treated with *racemic* and *meso* ^{59}Fe-*o,o*EDDHA separately did not lead to significant differences in Fe uptake,[22] whereas in Fe-deficient cucumber plants resupplied with Fe the final Fe shoot concentrations were 1.5-fold higher with *meso* than with *racemic* Fe-*o,o*EDDHA.[23]

The Fe tracers provided by both Fe(III)-*o,o*EDDHA diasteroisomers reached all plant tissues. Approximately 60% of the Fe tracers ($Fe^{ex\text{-}meso}$+$Fe^{ex\text{-}rac}$) taken up from Fe(III)-*o,o*EDDHA by Fe-deficient sugar beet were still localized in the fine roots at 3-6 h after Fe-resupply. At that time, leaves had incorporated only 12-17% (young leaves) and 22-25% (old leaves) of the total Fe tracers taken up. These results are in agreement with a previous study, where 80% of the Fe delivered to Fe-deficient cucumber plants by 100 µM of ^{57}Fe(III)-*o,o*EDDHA was also located in the roots 1 h after the treatment.[25] Average plant concentrations of Fe tracers ($Fe^{ex\text{-}meso}$ and $Fe^{ex\text{-}rac}$) were 0.6 and 1.1 µmol Fe g DW^{-1} in sugar beet (this study) and cucumber[25] plants, respectively. In leaves, the concentrations of Fe tracers were 2- to 4-fold higher in sugar beet (this study) than in tomato and peach treated for 18-28 d with 5 µM ^{57}Fe(III)-*o,o*EDDHA[26] (0.7-1.4 and 0.3 µmol g DW^{-1},

respectively); this could be associated to dilution effects during long term plant growth and/or to the different Fe concentrations applied. The distribution inside the plant of the Fe tracers ($Fe^{ex\text{-}meso}$ and $Fe^{ex\text{-}rac}$) was similar, with percentages of the Fe allocated in each tissue (fine roots : main roots : old leaves : young leaves, at 3-6 h after resupply) of 62-60 : 3-3 : 21-23 : 11-16 for the *meso* isomer and 55-56 : 3-4 : 23-27 : 13-19 for the *racemic* one. This indicates that there were no major differences in the plant allocation of the Fe delivered by both Fe-carriers ($Fe^{ex\text{-}meso}/Fe^{ex\text{-}rac}$ ratios in the range of 2.4-1.6), except for fine roots, where higher ratios (3.0-2.4) were found, indicating a preferential accumulation of $Fe^{ex\text{-}meso}$.

Iron-deficient sugar beet and tomato plants were found to incorporate the *o,o*EDDHA chelating agent in all tissues during Fe resupply, using a highly selective and sensitive HPLC-ESI/TOFMS method for the determination of *o,o*EDDHA.[16] This is in agreement with previous findings using HPLC-UV/Vis and ^{14}C-radioactive determinations.[9,12-15] The concentrations of *o,o*EDDHA found in sugar beet tissues (0.1-0.2 µmol g DW^{-1}) are within the range found in tomato (0.1-0.4 µmol g DW^{-1})[15,16], pepper (0.1-0.4 µmol g DW^{-1})[15] and tobacco plants (0.1-0.2 µmol g DW^{-1}).[14] These values are equivalent to approximately 30-70 µg *o,o*EDDHA g DW^{-1}. Approximately 80% of the chelating agent taken up was in the leaves after 6 h, in marked contrast with the 40% value found for Fe. This may suggest that *o,o*EDDHA is transported in the transpiration stream, in agreement with the hypothesis that plant uptake of the chelating agents (with or without the metal) occurs by absorption through openings in the root endodermis and the Casparian strip.[15,34] This type of absorption should be proportional to the time exposure of the plant to the chelate and the chelate concentration applied. In our study, the *o,o*EDDHA content in Fe-deficient sugar beet plants was only 1.5-fold higher on the average when the time of exposure doubled, with the highest increases being found in fine roots and young leaves, although the xylem concentrations did not change significantly. Also, whereas the Fe(III)-*o,o*EDDHA sugar beet and tomato xylem concentrations were similar at Fe(III)-*o,o*EDDHA concentrations below 500 µM, Fe(III)-*o,o*EDDHA concentrations above this limit caused significant increases (6-7 fold) in the Fe(III)-*o,o*EDDHA xylem concentrations in both species. These results may suggest that *o,o*EDDHA uptake is not fully explained by a simple passive mechanism, and that the occurrence of a rather complex process is more likely.

The *racemic*- and *meso*-*o,o*EDDHA isomers were in similar amounts (ratio of 1.0) in all tissues analyzed, irrespective of the resupply time (3-6 h) and chelate concentration (30-2000 µM Fe-*o,o*EDDHA). In a previous study using long-term applications to pepper and tomato plants, the *meso/racemic* ratios were slightly higher in leaves than in roots (approximately 1.0-1.4 and 1.0, respectively) and this was attributed to a preferential translocation of the *meso* isomer from roots to shoots and/or degradation of the *racemic* isomer in leaves.[15] Other authors observed that the *meso* Fe(III)-*o,o*EDDHA isomer disappeared in higher amounts from the nutrient solution when compared with the *racemic* one when Strategy I plants were used[20] suggesting that plants take up the *meso* isomer preferentially; however, in that study plant tissue *o,o*EDDHA was not quantified, and nutrient solution *racemic* and *meso* Fe(III)-*o,o*EDDHA was measured without considering that *o,o*EDDHA can be chelated with other metals after Fe release by the FCR.

The *o,o*EDDHA plant contents were one order of magnitude lower than those of Fe tracers ($Fe^{ex\text{-}meso}$+$Fe^{ex\text{-}rac}$) taken up from Fe(III)-*o,o*EDDHA. These results are in accordance with the values obtained with tomato and pepper plants[15], and support that the main mechanism of Fe acquisition from Fe(III)-*o,o*EDDHA by Fe-deficient Strategy I plants involves the release of Fe from the *racemic* and *meso o,o*EDDHA carriers. This

dissociative Fe acquisition mechanism was first demonstrated by Tiffin and Brown[12], and Römheld and Marschner[9], labeling Fe and *o,o*EDDHA with ^{55}Fe (or ^{59}Fe) and ^{14}C, respectively. The ratios ($Fe^{ex\text{-}meso}$+$Fe^{ex\text{-}rac}$)/*o,o*EDDHA differed among tissues (43-32, 10-50, 7-7 and 4-5 in fine roots, xylem, old leaves and young leaves, respectively, after 3-6 h of resupply), and point out to the preferential accumulation of Fe and chelating agent in roots and leaves, respectively. It is remarkable that the concentration of *meso* + *racemic* Fe(III)-*o,o*EDDHA accounted for 10 and 2% of the Fe tracers taken up ($Fe^{ex\text{-}meso}$+$Fe^{ex\text{-}rac}$) at 3 and 6 h, respectively. Also, ratios did not change much with resupply time, with the exception of the xylem, where the ratio ($Fe^{ex\text{-}meso}$+$Fe^{ex\text{-}rac}$)/(*racemic* + *meso* Fe(III)-*o,o*EDDHA) increased 5-fold between 3 to 6 h of resupply. This may suggest a differential uptake kinetics for Fe and chelating agent uptake, with a lag phase in the increase of xylem total Fe concentration when compared to that of Fe(III)-*o,o*EDDHA. The $Fe^{ex\text{-}meso}$/*meso-o,o*EDDHA ratio in the plant was approximately 2-fold higher than that of the $Fe^{ex\text{-}rac}$/*racemic-o,o*EDDHA, showing that the dissociative mechanism used by Fe-deficient sugar beet plants was more effective for the *meso* than for the *racemic* Fe(III)-*o,o*EDDHA isomer. The presence in the plants of significant amounts of chelating agents observed in this and other studies[13-16] opens the possibility that a small part of the Fe delivered by the Fe(III)-*o,o*EDDHA may be taken up *via* non-dissociative mechanisms. This may occur at all times, but the relative contribution of these mechanisms would be specially important both in the short term after Fe fertilization and also when root FCR activity is down-regulated by the Fe status (e.g., after fertilization as in López-Millán *et al.*[31] or in continuous low intensity Fe supply).

A 2.7-fold higher sugar beet root reduction rate was found for the *meso* isomer than for the *racemic* one, using the strong Fe(II) chelating agent BPDS to assess separately both stereoisomers; in a previous study a 7.5-fold increase was found with Fe-deficient cucumber roots.[23] The mechanism of Fe uptake from Fe(III)-chelates by Fe-deficient Strategy I plant species involves a reduction step,[35] followed by the spontaneous release or competitive sequestration of the reduced species.[4] A study on the reduction kinetics of various synthetic weak hexadentate polyaminocarboxylate Fe(III)-chelates (lacking phenolic groups, e.g. Fe(III)-EDTA and Fe(III)-DTPA) by Fe-deficient peanut roots showed that the differences in enzyme affinity (K_m) and maximal reduction rate (V_{max}) could be attributed to differences in chemical characteristics such as formation constant ($K_{Fe(III)L}$; $LogK_{Fe(III)L}$ in the range 18.2-31.2) and ionic charge (in the range 0 to -2).[36] However, the differences in these parameters between the *meso* and *racemic* Fe(III)-*o,o*EDDHA stereo-isomers are relatively small ($LogK_{Fe(III)L}$ of 34.2 and 35.9, respectively; charge of -1 in both cases), and therefore cannot explain the 3- to 8-fold differences in root reduction rate found here and in the Lucena and Chaney[23] study.

The differences in FCR reduction rates between *racemic* and *meso* Fe(III)-*o,o*EDDHA could be related to the pH dependence of the corresponding redox potentials. The cytoplasmatic reductant acting as electron donor for the root FCR is thought to be cytoplasmic NAD(P)H (midpoint potentials ($E^{0\prime}$) of approximately –320 mV)[37], with the Fe(III)-chelate, not Fe(III), being the electron acceptor.[38] The efficacy of the root FCR to release Fe from Fe(III)-chelates depends markedly on the redox potential in the cell surface environment.[39,40] The reduction of the complex Fe(III)-*o,o*EDDHA itself by the FCR enzyme has been recently questioned, because the very negative reduction potential (-560 mV at the pH 7.4 typical of calcareous soils[41] and the high spin Fe[42] of Fe(III)-*o,o*EDDHA make such reaction thermodynamically unfavorable. The same authors proposed that the electron acceptor of the FCR enzyme could be the Fe(III)-*o,o*EDDHA monoprotonated specie ([Fe(III)HL]) instead of the major $[Fe(III)L]^-$ species. In Fe-deficient Strategy I

plants, the induction of a root plasma membrane H^+-ATPase is a core component of the response to Fe deficiency, favoring the activity of the root FCR.[43] At lower pH values, the concentration of the protonated species increase, while the Fe(III)-*o,o*EDDHA redox potential increases to -480 and -370 mV at pH 6.0 and 5.0, respectively,[41] values closer to that of the electron donor. To the best of our knowledge, the redox properties of the individual *racemic* and *meso* Fe(III)-*o,o*EDDHA isomers have not been reported. However, the pH-dependence of the redox potential is determined by the protonation constants of the oxidized and reduced chelate forms[39,40], which are known for the *racemic* and *meso* Fe(III)-*o,o*EDDHA isomers. In the *meso* isomer the [Fe(III)HL] species was more stable than the $[Fe(III)L]^-$ species (LogK of 36.6 and 34.2, respectively), whereas in the *racemic* isomer the constants of the [Fe(III)HL] and $[Fe(III)L]^-$ species were similar (LogK of 35.1 and 35.9, respectively)[1]. The tendency of the *meso* isomer to form protonated species as the pH decrease when compared with the *racemic* one could be behind the higher relative efficiency of the *meso* isomer as an Fe source.

In the protonated species of *racemic* and *meso* Fe(III)-*o,o*EDDHA, *o,o*EDDHA coordinates with Fe as a pentadentate ligand. Iron(III)-chelate redox potentials depend on the first Fe coordination shell, which changes with ligand denticity (the number of atoms in a single ligand that bind to a central Fe atom in a coordination complex), among others[39]. For instance, the redox potential of 12 hydroxymate siderophore Fe(III)-complexes, with homologous binding groups but with different denticity, followed the sequence hexadentate>tetradentate>bidentate.[39] In the *racemic* and *meso* Fe(III)-*o,o*EDDHA $[FeL]^-$ species, the Fe(III) coordination arrangement is a six-coordinate, closed roughly octahedral field, and *o,o*EDDHA coordinates as a hexadentate ligand with 6 donor groups available for metal chelation (2 amino, 2 carboxylate and 2 phenolate groups). In the Fe(III)-*o,o*EDDHA [FeHL] species (that becomes more abundant when pH decreases), the arrangement is a six-coordinate, open octahedral field, and *o,o*EDDHA coordinates as a pentadentate ligand with a phenolic oxygen atom free and the vacant Fe coordination position occupied by a water molecule.[1] The Fe coordination arrangement of the Fe(III)-chelate has been hypothesized to be the origin of the better efficiency as a root FCR enzyme substrate (using BPDS and pH 6) of the regioisomer Fe(III)-*o,p*EDDHA when compared of Fe(III)-*o,o*EDDHA; the accessibility of the Fe atom for the FCR enzyme would be facilitated by the octahedral open form of Fe(III)-*o,p*EDDHA when compared to the octahedral closed form of the Fe(III)-*o,o*EDDHA $[FeL]^-$ species predominant at pH 6.[44] In fact, the Fe(III)-*o,o*EDDHA [FeHL] species has a first coordination shell similar to that of Fe(III)-*o,p*EDDHA, in both cases coordinating as pentadentate ligands.

The *meso* isomer appears to be the major contributor to the exceptional efficiency of Fe(III)-*o,o*EDDHA as a plant Fe source in nutrient solution (and therefore in similar conditions such as soil-less horticulture). However, both isomers behaved similarly as Fe sources in calcareous soil conditions,[22] where soil related factors can limit the efficiency of the *meso* isomer (e.g. by adsorption onto soil surfaces)[6,45] or metal and ligand exchange reactions). This is similar to what occurs with the Fe(III)-chelate of the pentadentate ligand *o,p*EDDHA, which was capable of providing sufficient Fe to plants in nutrient solution but not in calcareous soil.[26]

A rough comparison of rates of reduction and uptake can be made in the sugar beet experiment with Fe tracers. The amount of Fe tracer taken up ($Fe^{ex\text{-}meso}$+$Fe^{ex\text{-}rac}$) in leaves and the whole plant was approximately 11-15 and 40-62% of the Fe reduced in the presence of BPDS. The total Fe xylem concentrations, considering transpiration rates of 20 g water h^{-1} $plant^{-1}$,[46] would correspond to Fe transport rates equivalent to approximately

10% of the Fe reduced by the FCR; however, considering xylem Fe tracer concentrations, values would be equivalent to approximately 2% of the Fe reduced by the FCR. In previous studies, Fe-deficient peanut plants showed similar rates of Fe(III)-DTPA-reduction and ^{59}Fe-uptake,[47] whereas in Fe-deficient cucumber plants the xylem Fe concentration accounted for only 1% of the Fe reduced in the BPDS assay.[23] These results point out to the need for a system to assess physiological Fe reduction rates directly, in the absence of strong Fe(II) chelators such as BPDS, which can lead to overestimations of the reduction rates.[48]

5. CONCLUSIONS

The usefulness of dual-stable Fe isotope tracer experiments in plant Fe nutrition has been proven, since plants did not discriminate between ^{54}Fe and ^{57}Fe and no short-term Fe isotope exchange reactions occurred with the Fe(III)-chelates used. The *meso* isomer appears to be the major contributor to the exceptional efficiency of Fe(III)-*o,o*EDDHA to deliver Fe to plants in nutrient solution, with rates of FCR, xylem transport and total uptake (in both cases considering the Fe previously chelated) 2-fold higher than those found for the *racemic* isomer. Both isomers of the chelating agent were incorporated and distributed by plants at similar rates, in amounts one order of magnitude lower than those of Fe. After 6 h of Fe resupply, most of the Fe acquired was still localized in roots, whereas most of the chelating agent was localized in leaves. Although most of the Fe coming form the Fe(III)-*o,o*EDDHA was taken up by the plant through a dissociative reduction mechanism, a small part of the Fe delivered by the Fe(III)-*o,o*EDDHA may be taken up *via* non-dissociative mechanisms, probably using the transpiration stream as the driving force for entry, and this may be important in the short term after Fe fertilization and also when root FCR activity is down-regulated.

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Capítulo 7

DISCUSIÓN GENERAL

Este trabajo de investigación ha consistido en el desarrollo y aplicación de nuevos métodos analíticos basados en la espectrometría de masas (MS) que permitan la caracterización de fertilizantes férricos basados en quelatos sintéticos y la determinación, tanto del Fe como del agente quelante, en diferentes matrices agrícolas. Se ha escogido la técnica de espectrometría de masas con fuente de electrospray y analizador de tiempo de vuelo ESI-MS(TOF) para la caracterización de productos comerciales y la cuantificación del quelato, debido a la alta selectividad de la relación masa/carga (*m/z*) de la molécula, la alta resolución del analizador TOF, que permite ver la huella isotópica del metal, y su elevada sensibilidad. Esta misma técnica, pero con espectrometría de masas en tándem (ESI-MS-MS(QTOF)), ha sido utilizada para estudiar los patrones de fragmentación de los quelatos férricos utilizados como fertilizantes. Esta información ha servido para la identificación de compuestos de Fe desconocidos presentes en fertilizantes comerciales. Un tercer tipo de MS, la de plasma de acoplamiento inductivo (ICP-MS), ha sido la elegida para la cuantificación del Fe dentro de la planta, debido a la alta sensibilidad de la técnica y su capacidad para diferenciar isótopos estables de Fe, lo que ha permitido realizar un seguimiento del Fe aportado por dos fertilizantes simultáneamente. El uso combinado de estas tres técnicas de MS junto con la cromatografía líquida de alta eficacia (HPLC) ha permitido desvelar nuevas implicaciones biológicas derivadas del uso de quelatos férricos sintéticos y abrir nuevas aproximaciones analíticas para futuros estudios medio ambientales y biológicos.

En esta Tesis Doctoral se ha desarrollado un método altamente selectivo para determinar simultáneamente los quelatos férricos sintéticos utilizados como fertilizantes (Fe(III)-EDTA, Fe(III)-DTPA, Fe(III)-HEDTA, Fe(III)-CDTA, Fe(III)-*o,o*EDDHA, Fe(III)-*o,p*EDDHA, Fe(III)-*o,o*EDDHMA, Fe(III)-EDDHSA y Fe(III)-EDDCHA) mediante HPLC-ESI-MS(TOF) (Álvarez-Fernández, Orera *et al*., 2007). Para cada quelato se utilizó como estándar interno (IS) su homólogo unido al isótopo estable ^{57}Fe, ya que coeluyen cromatográficamente con los analitos y presentan similares efectos de ionización. La sensibilidad alcanzada en este método fue buena, con límites de detección (LOD) entre 3 y 164 pmol dependiendo del quelato (0.1-3.3 µM). Esta sensibilidad es mejor que la de otros métodos analíticos previamente publicados con cromatografía de par iónico y detección por UV-vis como el de Deacon en 1994 con un LOD de 1790 pmol (Deacon *et al.*, 1994) o el de Lucena en 1996 capaz de determinar concentraciones de quelato por encima de 9 µM (Lucena *et al.*, 1996). Sin embargo, se obtuvieron mejores LOD con la técnica de HPLC-ICP-MS ya que, por lo general, es más sensible que la de HPLC-ESI-MS, llegando a alcanzar un LOD para el Fe(III)-EDTA de 125-150 nM (Ammann, 2002a). La respuesta del detector fue lineal para todos los quelatos férricos en el intervalo de concentraciones del límite de cuantificación (LOQ) hasta 25-50 µM con valores de R de 0.9962–0.9997. La reproducibilidad del método para los tiempos de retención fue siempre inferior al 2% (desviación estándar relativa; RSD) mientras que para las áreas fue del 5-8%. Estos valores están dentro del rango obtenido para otros métodos desarrollados con HPLC-ESI-MS (Dodi y Monnier, 2004) y HPLC-ICP-MS (Ammann, 2002a) pero son considerablemente mayores que los obtenidos con HPLC-UV-vis, que están en el rango del 1% de RSD (Barak y Chen, 1987; Boxema, 1979; García-Marco *et al.*, 2006).

El aspecto probablemente más interesante y novedoso de este método analítico fue su validación para cada uno de los quelatos individualmente en cuatro matrices agrícolas líquidas: solución del suelo, solución nutritiva, agua de riego y savia de xilema de melocotonero. Los valores de ensayos de recuperación obtenidos para estas matrices fueron buenos en el intervalo de 82-100% para todas las matrices excepto para la savia de xilema,

donde se obtuvieron valores de 70-111%. Hasta este momento sólo existían métodos para determinar algunos quelatos férricos sintéticos en aguas subterráneas y lixiviados (Amman 2002a, 2002b), en aguas industriales (Dodi y Monnier, 2004) y residuales (Knepper *et al.*, 2005) y en algún tejido vegetal (Batra y Maier, 1964; Jeffreys y Wallace, 1968; Bienfait *et al.*, 2004).

Este método de HPLC-ESI-MS(TOF) fue aplicado al análisis del contenido de quelato férrico en siete fertilizantes comerciales de Fe(III)-EDTA, Fe(III)-DTPA, Fe(III)-HEDTA, Fe(III)-EDDHA y Fe(III)-EDDHMA. El contenido de Fe ligado al agente quelante en estos fertilizantes fue del orden de los publicados por García-Marco (2005a) en un estudio en el que se analizó una muestra numerosa de estos fertilizantes aplicando los métodos oficiales de análisis de la Unión Europea. En esta Tesis los contenidos de quelato férrico representaron un 47-107% del contenido de Fe soluble indicado en la etiqueta del fertilizante. Este hecho, que ya había sido reflejado anteriormente (García-Marco, 2005a), indica la presencia de una importante fracción de Fe soluble unido a compuestos diferentes al quelato sintético autorizado por la legislación europea de fertilizantes (EC Nº 2003/2003; Anon, 2003, EU Regulations Nº 2076/2004; Anon, 2004, EU Regulations Nº 162/2007; Anon, 2007). Estos productos de Fe son generalmente desconocidos, aunque algunos de ellos se han identificado como isómeros posicionales (Fe(III)-*o,p*EDDHA, Fe(III)-*o,p*EDDHMA) o productos de condensación (derivados del Fe(III)-EDDHSA). Esta identificación se llevó a cabo mediante técnicas de HPLC-UV-vis, 1D-, 2D-RMN y ESI-MS (Álvarez-Fernández *et al.*, 2002; Cremonini *et al.*, 2001; García-Marco *et al.*, 2005b; Gómez-Gallego *et al.*, 2002). En esta Tesis Doctoral se ha estudiado la utilidad de la técnica de HPLC-ESI-CID-MS^2(QTOF) como herramienta para identificar los compuestos de Fe desconocidos presentes en fertilizantes comerciales. Para ello se estudiaron los espectros de fragmentación ESI-CID-MS^2 de los estándares de cada uno de los quelatos férricos. Los espectros ESI-CID-MS^2 se obtuvieron con dos analizadores de masas diferentes, una trampa iónica y un cuadrupolo-tiempo de vuelo (QTOF), siendo este último el que mayor riqueza estructural proporcionó, permitiendo incluso diferenciar patrones de fragmentación de isómeros posicionales como el Fe(III)-*o,o*EDDHA y el Fe(III)-*o,p*EDDHA sin necesidad de una separación cromatográfica previa. Sin embargo, los estereoisómeros *racémico* y *meso* del Fe(III)-*o,o*EDDHA y Fe(III)-*o,o*EDDHMA no se pudieron diferenciar por MS^2. Las descarboxilaciones fueron las fragmentaciones comunes para todos los quelatos. Sin embargo, dependiendo del quelato, se detectaron diferentes pérdidas de masa así como diferentes intensidades relativas en las pérdidas de masa comunes. Por ejemplo, en la mayoría de los quelatos, el fragmento más intenso correspondió a pérdidas de los grupos carboxilo salvo para el Fe(III)-CDTA, Fe(III)-*o,p*EDDHA, Fe(III)-EDDCHA y Fe(III)-EDDHSA donde los iones producto más intensos correspondieron a pérdidas en el anillo. Los espectros de fragmentación de los quelatos férricos sintéticos sólo existían en la bibliografía para dos casos, el Fe(III)-EDTA y el Fe(III)-DTPA (Quintana y Reemtsma, 2007).

Cuando se analizó un fertilizante comercial a base de Fe(III)-EDDHA mediante HPLC-ESI-CID-MS^2(QTOF) con el fin de probar la aplicabilidad de esta técnica para identificar compuestos de Fe desconocidos, se detectaron gracias a la huella isotópica dos compuestos férricos. La identificación se abordó comparando sus masas moleculares exactas y espectros de fragmentación con los de los estándares de Fe(III)-*o,o*EDDHA y el Fe(III)-*o,p*EDDHA. De esta manera se propusieron dos identidades para ambos compuestos (figuras 6-5A y 6-5B) que pueden ser explicadas como subproductos de la síntesis del agente quelante EDDHA, que se producen por reacción con impurezas de los productos de partida etilendiamina y ácido glioxílico (Boichard *et al.*, 1971; Deeba *et al.*, 1990). Por

consiguiente, esta herramienta podría ser muy útil para conocer la identidad de una gran parte de la fracción soluble de Fe presente en fertilizantes comerciales a base de quelatos sintéticos.

Ser capaces de determinar los agentes quelantes sintéticos dentro de la planta fue uno de los objetivos de esta Tesis Doctoral debido a las implicaciones biológicas que conlleva encontrar estas moléculas xenobióticas dentro de la planta. La determinación de este tipo de moléculas en matrices vegetales sólidas es complejo debido al elevado número de compuestos que pueden afectar a la ionización del analito, las bajas concentraciones esperadas y a la variedad de especies químicas en las que se puede encontrar el analito dentro de los tejidos vegetales. Por ello, se desarrolló un método de HPLC-ESI-MS(TOF) para matrices vegetales exclusivamente para el agente quelante *o,o*EDDHA ya que es el más utilizado en suelos calizos donde muchos cultivos deben ser fertilizados con Fe. Un aspecto crítico del desarrollo de este método fue la elección del IS. Contrariamente a lo sucedido en el método anterior, los IS marcados con isótopos estables de Fe (^{57}Fe(III)-*o,o*EDDHA y ^{54}Fe(III)-*o,o*EDDHA) no dieron buenos resultados debido a la presencia de interferencias isobáricas y al intercambio isotópico producido entre el Fe marcado isotópicamente del IS y el Fe nativo del tejido. Los mejores estándares internos son aquellos marcados isotópicamente en C, N u O, sin embargo suelen resultar excesivamente caros. Una buena alternativa a los IS marcados con isótopos estables son los análogos estructurales, ya que poseen una estructura muy similar al analito y generalmente un comportamiento químico muy parecido al de éste. El análogo estructural que se utilizó fue el compuesto metilado del *o,o*EDDHA (*o,o*EDDHMA) que tiene un grupo metilo unido a cada anillo aromático. De los dos isómeros del Fe(III)-*o,o*EDDHMA, se eligió el más cercano en tiempo de retención al Fe(III)-*o,o*EDDHA que coeluía con el Fe(III)-*meso*-*o,o*EDDHA y tenía una diferencia de tan sólo 2 min con el Fe(III)-*racémico*-*o,o*EDDHA. Esta diferente elución del IS y el *racémico* Fe(III)-*o,o*EDDHA afectó a la corrección de los efectos matriz que no fue completa en las relaciones de material vegetal / extracto final inicialmente elegidas. Por este motivo, estas relaciones se disminuyeron para cada tejido vegetal hasta que se obtuvo un valor de recuperación próximo al 100%. A pesar de la dilución que se tuvo que hacer para algunos tejidos vegetales, la sensibilidad del método es muy buena siendo la concentración mínima de *o,o*EDDHA que se puede cuantificar en tejidos vegetales de 11-162 pmol g^{-1} peso fresco (FW) y en savia de xilema 60-151 pmol mL^{-1}. Estos límites de cuantificación (LOQ) son al menos un orden de magnitud mejores que el único LOQ publicado para medir *o,o*EDDHA en tejido vegetal mediante HPLC-UV/Vis (2140 pmol g^{-1}FW; Bienfait *et al.*, 2004). Otro aspecto importante que hubo que tener en cuenta para el desarrollo de este método fue el hecho de que los agentes quelantes pueden encontrarse unidos a otros metales diferentes del Fe dentro de la planta. Por este motivo, se añadió un exceso de Fe a los extractos vegetales antes de ser analizados, de manera que las posibles especies de *o,o*EDDHA se convirtieran en una única especie de Fe y poder ser cuantificado en su totalidad. Los ensayos de recuperación obtenidos con esta metodología en distintos tejidos (raíz, hoja, savia de xilema y fruto) y especies vegetales (tomate, remolacha azucarera y melocotonero) fueron buenos, del orden de 74-118%. Este método analítico de HPLC-ESI-MS(TOF) fue aplicado al análisis de hojas, raíces y savia de xilema de plantas de remolacha azucarera y tomate tratadas hidropónicamente con un fertilizante comercial de Fe(III)-EDDHA. En todos los tejidos analizados se encontraron los dos grupos de estereoisómeros del Fe(III)-*o,o*EDDHA (*racémico* y *meso*) y el isómero Fe(III)-*o,p*EDDHA, que está también presente en las formulaciones comerciales de Fe(III)-EDDHA (aproximadamente en un 1% p/p ; Álvarez-Fernández, Orera *et al.*, 2007). Las concentraciones de *racémico* y *meso*-*o,o*EDDHA encontradas en este trabajo (12-63 nmol

g^{-1} FW, Tabla 5-1) son parecidas a las encontradas en trabajos anteriores (Bienfait *et al.*, 2004; Jeffreys y Wallace, 1968) a pesar de que nuestros tiempos de tratamiento fueron menores. Hay que remarcar también que este es el primer trabajo en el que se observa la presencia del isómero Fe(III)-*o,p*EDDHA dentro de la planta. Sin embargo, no ha sido posible su cuantificación debido a la falta de estándares.

En esta Tesis Doctoral se ha utilizado un método de doble marcaje con dos isótopos estables de Fe (^{54}Fe y ^{57}Fe) para estudiar la toma, transporte y distribución del Fe proveniente de los isómeros *racémico* y *meso* Fe(III)-*o,o*EDDHA cuando se aplican simultáneamente a las raíces de plantas de Etrategia I deficientes en Fe crecidas en hidroponía. Después de 3 y 6 horas de tratamiento los isótopos estables de Fe fueron analizados por ICP-MS y los agentes quelantes por HPLC-ESI-MS(TOF). No se observaron reacciones de intercambio entre isótopos de la solución nutritiva y tampoco discriminación isotópica por parte de la planta. El mayor aporte de Fe a la planta lo realizó el isómero *meso* del Fe(III)-*o,o*EDDHA, ya que se encontró 2 vecés más de ese Fe dentro de la planta ($Fe^{ex\text{-}meso}$) que el del proveniente del *racémico* ($Fe^{ex\text{-}rac}$). Este hecho concuerda bien con los experimentos realizados en solución nutritiva de plantas de judía, tomate y pimiento en las que se observaba una preferente disminución del Fe proveniente del isómero *meso* en la solución nutritiva, lo que parecía indicar que este Fe era tomado por la planta preferentemente (Cerdán *et al.*, 2006; Hill-Cottingham y Lloyd-Jones, 1965). Además, las velocidades de reducción del Fe(III) a Fe(II) por la enzima quelato reductasa férrica (FCR) medidas en nuestro estudio indican una mayor reducción del isómero *meso*-*o,o*EDDHA (casi 3 veces) que el *racémico*, lo que explicaría los mayores contenidos de $Fe^{ex\text{-}meso}$ encontrados dentro de la planta. Este mismo comportamiento se observó en estudios previos de pepino con una diferencia de 7 veces entre el isómero *meso* y el *racémico* (Lucena y Chaney, 2006).

El agente quelante *o,o*EDDHA se encontró en todos los tejidos de la planta analizados, como indicaron estudios previos de HPLC-UV-vis y ^{14}C (Bienfait *et al.*, 2004; Jeffreys y Wallace, 1968; Römheld y Marschner, 1981). Sin embargo, la presencia de estos xenobióticos no había sido demostrada con técnicas analíticas tan selectivas como la MS hasta esta Tesis Doctoral en la que la entrada de ambos isómeros a las plantas de Estrategia I (remolacha y tomate) se confirma con los análisis de HPLC-ESI-MS(TOF). Las concentraciones de *o,o*EDDHA encontradas en los tejidos de remolacha (0,1-0,2 µmol g peso seco $(DW)^{-1}$) son similares a las encontradas previamente en tomate, pimiento (0,1-0,4 µmol g DW^{-1}; Bienfait *et al.*, 2004) y tabaco (0,1-0,2 µmol g DW^{-1}; Jeffreys y Wallace, 1968). Ambos isómeros del *o,o*EDDHA (*racémico* y *meso*) fueron incorporados y distribuidos en la planta en la misma proporción, siendo las cantidades de agente quelante encontradas un orden de magnitud menor que la del Fe. Este resultado es similar al encontrado en plantas de tomate y pimiento (Bienfait *et al*. 2004) y es congruente con el mecanismo de disociación del Fe del quelato para la adquisición del Fe proveniente de Fe(III)-*o,o*EDDHA en plantas de Etrategia I deficientes en Fe. Este mecanismo de disociación fue demostrado por primera vez por Tiffin y Brown (1961) y Römheld y Marschner (1981) marcando *o,o*EDDHA con ^{55}Fe (o ^{59}Fe) y ^{14}C. Sin embargo, la presencia de cantidades pequeñas de agente quelante dentro de la planta abre la posibilidad de que una pequeña parte del Fe sea incorporado a la planta mediante mecanismos que no incluyen disociación. Este mecanismo de entrada de Fe a la planta sería especialmente importante en fertilizaciones de Fe a corto plazo y cuando la actividad de la enzima FCR fuera baja (como por ejemplo en fertirrigaciones continuadas con bajas cantidades de Fe o varias horas después de una fertilización).

Después de 6 h de fertilización, la mayoría del Fe absorbido se encuentra localizado en las raíces secundarias de la planta, mientras que el agente quelante se encuentra principalmente en las hojas. Una distribución similar se describió en plantas de pepino tratadas hidropónicamente con 100 µM de ^{57}Fe(III)-*o,o*EDDHA donde el 80% del Fe tomado se encontraba en las raíces de las plantas (Rodríguez-Castrillón *et al.*, 2008). Este hecho nos hace sugerir que el *o,o*EDDHA es transportado por la corriente de transpiración, de acuerdo con la hipótesis de que la toma de agentes quelantes (unidos o no al metal) ocurre por absorción pasiva a través de aperturas en la endodermis y banda de Caspari de la raíz (Bienfait *et al.*, 2004; Nowack *et al.*, 2006). Este tipo de absorción debería ser proporcional al tiempo de exposición de la planta al quelato y a la concentración de quelato aplicada. En nuestro estudio, el contenido de *o,o*EDDHA en la planta se incrementó sólo 1,5 veces cuando el tiempo de exposición al agente quelante aumentó 2 veces, encontrándose los mayores incrementos en las raíces secundarias y hojas jóvenes. Además, cuando plantas de remolacha y tomate fueron tratadas con diferentes concentraciones de Fe(III)-*o,o*EDDHA en solución nutritiva se observó que concentraciones inferiores a 500 µM no tuvieron efecto en la concentración de Fe(III)-*o,o*EDDHA encontrada en la savia de xilema. Sin embargo, concentraciones de Fe(III)-*o,o*EDDHA superiores a 500 µM en la solución nutritiva causaron incrementos de 6-7 veces en la concentración de este quelato en xilema. Estos resultados sugieren que la entrada de *o,o*EDDHA a las plantas no se puede explicar en su totalidad por un mecanismo de absorción pasivo.

En definitiva, la técnica de espectrometría de masas en sus diferentes modalidades ha resultado de gran utilidad en el estudio de los quelatos férricos sintéticos permitiendo la determinación simultanea de todos los quelatos férricos a bajas concentraciones en matrices agrícolas (ESI-MS), la elucidación estructural de impurezas en fertilizantes comerciales (CID-MS^2) y el seguimiento del Fe (ICP-MS) y del agente quelante (ESI-MS) aportado por dos fertilizantes simultáneamente dentro de la planta. Esta Tesis Doctoral muestra la utilidad de estas técnicas en el área de Ciencias Agrarias abriendo nuevas aproximaciones para estudiar la nutrición de Fe (y otros micronutrientes) en plantas, sobre todo en relación con los compuestos naturales implicados en la adquisición y transporte de estos elementos en la planta. Estos aspectos ya fueron explorados en dos colaboraciones realizadas en el marco de esta Tesis Doctoral, en una de ellas se logró identificar el $Fe(III)_3Citrato_3$ como responsable del transporte de Fe en plantas de tomate (ver publicación en Anexo 2) ayudando a avanzar en el entendimiento del transporte a larga distancia de este metal en plantas. Esta identificación se logró utilizando la combinación de HPLC-ICP-MS, HPLC-ESI-MS e isótopos estables de Fe. Otra aplicación más centrada en el campo de la agricultura, fue una primera aproximación al estudio de las posibles interacciones entre quelato y surfactante en fertilización foliar de hierro por ESI-MS (ver publicación en Anexo 2). Igualmente las técnicas de MS utilizadas en esta Tesis Doctoral así como las metodologías desarrolladas y las aproximaciones experimentales empleadas parecen prometedoras para abordar la caracterización de fertilizantes de micronutrientes a base de compuestos orgánicos tanto sintéticos como naturales así como su determinación en matrices complejas como las medioambientales.

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Capítulo 8

RESUMEN

Los fertilizantes a base de quelatos férricos sintéticos son xenobióticos de elevado precio comúnmente utilizados para corregir o prevenir uno de los desórdenes nutricionales más habituales de las plantas, la deficiencia de Fe. Estas moléculas son derivados de ácidos poliaminocarboxílicos (por ejemplo EDTA) con alta afinidad por el Fe y otros metales. Su uso con fines agrícolas o industriales está siendo cuestionado, ya que son difícilmente degradables, muy persistentes en el medio ambiente y pueden alterar el equilibrio natural de los metales (nutrientes o tóxicos) en el suelo, haciendo por ejemplo más biodisponibles determinados metales hasta alcanzar niveles tóxicos. Los estudios de estos quelatos en el medio ambiente son aún escasos, debido a la falta de metodologías analíticas capaces de determinar concentraciones muy bajas de los mismos en matrices tan complejas como el sistema suelo-planta. Sin embargo, las formulaciones comerciales a base de quelatos férricos sintéticos sí se han estudiado en mayor profundidad, sobre todo en la última década, debido a que la complejidad de su análisis es menor y al interés de la Unión Europea por regular la comercialización de estos compuestos. Existen diversos métodos analíticos para determinar el contenido de quelatos férricos en formulaciones comerciales, que son específicos para uno o varios agentes quelantes sintéticos. Sin embargo, hasta ahora, no existía un método analítico que permitiera determinar de manera simultánea todos los quelatos férricos sintéticos autorizados por la legislación europea. Por otra parte, se ha encontrado un desacuerdo entre el contenido de Fe soluble y el del quelato férrico sintético autorizado, llegando a haber en el caso de quelatos de tipo fenólico un 40-50% de Fe soluble no unido al quelato (García-Marco 2005), lo que ha propiciado diversos cambios en la legislación (EC Nº 2003/2003; Anon, 2003, EU Regulations Nº 2076/2004; Anon, 2004, EU Regulations Nº 162/2007; Anon, 2007). Los compuestos diferentes al quelato autorizado a los que se encuentra unido el Fe soluble son poco conocidos, aunque en ciertos trabajos se ha identificado alguno de ellos por cromatografía de alta eficacia y espectroscopía ultravioleta-visible (HPLC-UV-vis), resonancia magnética nuclear (RMN) y espectrometría de masas con electrospray (ESI-MS). La identificación de estos compuestos resulta muchas veces compleja debido a que se trata de mezclas de varios compuestos a diferentes concentraciones, a la necesidad de eliminar el Fe para su análisis por técnicas como la RMN y a la falta de estándares que permitan confirmar su estructura. Además, a pesar de que el uso de quelatos férricos sintéticos esta muy extendido en agricultura, el estudio de la absorción, transporte y distribución del Fe y el agente quelante dentro de la planta ha sido abordado en escasas ocasiones. En todas ellas, se han utilizado metodologías analíticas que no permiten diferenciar simultáneamente y de forma selectiva el Fe aportado por el fertilizante y el Fe nativo de la planta, ni llevar a cabo la detección selectiva de los agentes quelantes en matrices tan complejas como los tejidos vegetales. De todos los quelatos férricos sintéticos que se comercializan en el mercado, aquellos basados en el quelato Fe(III)-*o,o*EDDHA son los más utilizados, sobre todo en cultivos de alto valor añadido crecidos sobre suelos calizos como los de Aragón. Por otro lado, el Fe(III)-*o,o*EDDHA está compuesto por una mezcla de isómeros (*d,l-racémico* y *meso*) cuyo comportamiento ha sido poco estudiado. Por último, la espectrometría de masas es una técnica con alta sensibilidad y selectividad que ha sido aplicada con éxito en la determinación de compuestos organometálicos. La gran versatilidad que ofrecen los equipos existentes permitiendo el análisis a nivel atómico y molecular junto con la determinación de isótopos estables, hacen de la MS una herramienta con gran potencial en las determinaciones derivadas de estudios biológicos o medioambientales

1. OBJETIVOS:

Por todo lo anterior, los objetivos específicos de esta Tesis Doctoral son los siguientes:

1. Desarrollo y validación de un método analítico para la determinación de quelatos de Fe sintéticos en matrices agrícolas líquidas mediante HPLC-ESI-MS.

2. Desarrollo y validación de una metodología para la determinación del agente quelante de Fe, el *o,o*EDDHA, en tejidos vegetales mediante HPLC-ESI-MS.

3. Estudio de los patrones de fragmentación de quelatos férricos sintéticos mediante CID-ESI-MS/MS y su aplicación como herramienta para la caracterización de la fracción soluble de fertilizantes comerciales a base de estos quelatos.

4. Estudio de la absorción, transporte y distribución del Fe y de los agentes quelantes aportados por los isómeros *racémico* y *meso* del fertilizante Fe(III)-*o,o*EDDHA en plantas de Estrategia I.

2. METODOLOGÍA:

2.1 Quelatos férricos. Objetivos 1, 2, 3 y 4

Los quelatos férricos de EDTA, DTPA, *o,o*EDDHA, CDTA y EDDHMA se sintetizaron con Fe natural, ^{57}Fe y ^{54}Fe añadiendo soluciones ácidas de Fe sobre el agente quelante disuelto siguiendo el procedimiento descrito por Lucena *et al.* (1996). Debido a la falta de estándares comerciales de los agentes quelantes EDDHSA, EDDCHA y *o,p*EDDHA, los quelatos de Fe(III)-EDDHSA y Fe(III)-EDDCHA fueron cedidos por el profesor J. M. García-Mina (Universidad de Navarra) y el agente quelante *o,p*EDDHA fue cedido por el profesor J. J. Lucena (Universidad Autónoma de Madrid). Los isómeros *racémico* y *meso* del *o,o*EDDHA se separaron siguiendo el procedimiento descrito en Yunta *et al.* (2003). Además se utilizaron fertilizantes comerciales de EDTA, DTPA, EDDHA, EDDHMA y EDDHSA.

2.2 Material vegetal. Objetivos 1, 3 y 4

Las especies vegetales utilizadas en esta Tesis han sido remolacha (*Beta vulgaris L.*), tomate (*Lycopersicon esculentum L.*) y melocotonero (*Prunus persica L.*). De remolacha y tomate se muestrearon por separado las hojas jóvenes y viejas, raíces y savia se xilema. De melocotonero se muestrearon las hojas y frutos. Las plantas de tomate y remolacha se obtuvieron mediante cultivos hidropónicos en cámara de cultivo. El material vegetal de melocotonero se muestreó directamente de parcelas comerciales de la provincia de Zaragoza. La savia de xilema de remolacha y tomate se recogió mediante centrifugación de peciolos o decapitación del tallo, respectivamente, según los procedimientos descritos por López-Millán *et al.* (2000 y 2009).

2.3 Instrumentación. Objetivos 1, 2, 3 y 4

Espectrómetro de masas con analizador de tiempo de vuelo (BioTOF II o MicrOTOF, Bruker Daltonics) equipado con una fuente de electrospray y acoplado a un HPLC (Alliance 2795, Waters).

Espectrómetro de masas con fuente de plasma de acoplamiento inductivo equipado con celda de colisión (Q-ICP-MS 7500 ce, Agilent Technologies).

Espectrómetro de masas en tándem con con analizadores cuadrupolo y tiempo de vuelo y equipado con fuente de electrospray (QTOF, Bruker Daltonics) acoplado a un HPLC (1100 HPLC system, Agilent Technologies).

Espectrómetro de masas con analizador de trampa de iones esférica y fuente de electrospray (HCT Ultra, Bruker Daltonics) acoplado a un HPLC (Alliance 2795, Waters).

2.4 Extracción de *o,o*EDDHA de los tejidos vegetales. Objetivos 2 y 4

Se llevó a cabo mediante una extracción sólido-líquido, tomando como base el método descrito por Bienfait *et al.* (Bienfait *et al.*, 2004). Se optimizaron varios parámetros relacionados con la molienda, la centrifugación, el estándar interno y la relación peso de material vegetal/peso del extracto.

2.5 Digestión ácida de materiales vegetales y soluciones nutritivas. Objetivo 4

Los tejidos vegetales se secaron en una estufa a 60 ºC y se molieron en un molino de bolas (MM301, Retsch). La digestión de los tejidos vegetales se realizó en un digestor de microondas (Ethos 1, Millestone) con HNO_3 y H_2O_2. La savia de xilema y soluciones nutritivas se digerieron añadiendo HNO_3 al 1%.

2.6 Desarrollo y validación de los métodos. Objetivos 1 y 2

El desarrollo del método de HPLC-ESI-MS para determinar quelatos férricos sintéticos en matrices agrícolas (objetivo 1) consistió en la optimización de los parametros de ionización por electrospray así como selección de la fase estacionaria, fase móvil, modo de elución, flujo y volumenes de inyección más apropiados para la determinación simultánea de todos los quelatos férricos. Este método se validó para cada quelato de Fe obteniendo i) curvas de calibrado con estándar interno, ii) límites de detección (LODs) y cuantificación (LOQs), iii) reproducibilidad del método entre días y entre réplicas a dos concentraciones distintas y iv) ensayos de recuperación para cuatro matrices agrícolas líquidas (solución del suelo, agua de riego, solución nutritiva y fluido vegetal).

La metodología para determinar *o,o*EDDHA en tejidos vegetales mediante HPLC-ESI-MS (objetivo 2) se desarrolló mediante ensayos de recuperación del *o,o*EDDHA pre- y post-extracción con diferentes estándares internos y diferentes tejidos vegetales. Se eligió el estándar interno que era capaz de controlar mejor los efectos matriz. Se optimizó la relación peso de material vegetal/peso final del extracto para cada tejido vegetal. Se determinaron los límites de detección y cuantificación y se realizaron ensayos de recuperación del analito para cada material vegetal.

2.7 Caracterización de quelatos férricos sintéticos por CID-MS-MS. Objetivo 3

Soluciones de estándares y fertilizantes comerciales se analizaron por inyección directa en dos equipos de espectrometría de masas en tándem, i) QTOF y ii) trampa de iones. Los patrones de fragmentación de los quelatos férricos sintéticos fueron descritos a partir de los espectros obtenidos con el QTOF atendiendo a su masa molecular exacta y entorno isotópico, así como al análisis de los mismos quelatos sintéticos marcados con ^{57}Fe y con ayuda de la herramienta informática Smart Formula (Data Analysis v 4.0, Bruker Daltonics). La identificación de nuevas impurezas en fertilizantes comerciales se llevó a cabo primero haciendo un barrido en HPLC-ESI-MS de posibles compuestos con huella isotópica de Fe y segundo obteniendo el espectro de CID-MS^2(QTOF) previa separación por HPLC de las trazas (*m/z*) desconocidas que contenían Fe.

2.8 Estudio de la utilización del fertilizante Fe(III)-*o,o*EDDHA por plantas de Estrategia I. Objetivo 4

Plantas de tomate y remolacha deficientes en Fe fueron tratadas en solución nutritiva con diferentes concentraciones de Fe-*o,o*EDDHA (una mezcla 1:1 de *racémico* y *meso*) durante 24 h y se midió su concentración en Fe y *o,o*-EDDHA en savia de xilema. Además, los isómeros *racémico* y *meso-o,o*EDDHA fueron marcados con dos isótopos estables de Fe diferentes (^{57}Fe y ^{57}Fe) y se suministraron simultáneamente y en igual concentración, a plantas de remolacha deficientes en Fe crecidas en hidroponía durante 3 y 6 h. Se hicieron

dos tratamientos cruzados: (A) 30 µM de ^{54}Fe-*racémico-o,o*EDDHA y 30 µM de ^{57}Fe-*meso-o,o*EDDHA y (B) 30 µM de ^{57}Fe-*racémico-o,o*EDDHA y 30 µM de ^{54}Fe-*meso-o,o*EDDHA. Se determinó el contenido de ambos isómeros de *o,o*EDDHA y del Fe proveniente del isómero *racémico* y *meso* Fe(III)-*o,o*EDDHA, así como el ^{nat}Fe (Fe natural) presente en plantas de remolacha deficientes en Fe tratadas durante 3 y 6 horas con ambos tratamientos (A y B). El contenido de *o,o*EDDHA se determinó en diferentes tejidos de la planta con método de HPLC-ESI-MS desarrollado en el objetivo 2. El contenido en Fe enriquecido isotópicamente (^{54}Fe y ^{57}Fe) y ^{nat}Fe en los diferentes tejidos de la planta se determinó por análisis de dilución isotópica mediante ICP-MS (Rodríguez-Castrillón *et al*., 2008). Además se midió la actividad Fe-reductasa de raíz de plantas de remolacha deficientes en Fe, utilizando separadamente *racémico* y *meso* Fe(III)-*o,o*EDDHA como sustratos. Para ello se midieron las concentraciones de un complejo con Fe(II), el [Fe(II)-$BPDS_3$], en soluciones nutritivas a diferentes tiempos de reacción (30 min, 1 h, 2 h y 5 h), tal y como indican Lucena y Chaney (Lucena y Chaney, 2006).

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3. CONCLUSIONES

1. Se ha desarrollado un método de HPLC-ESI-MS(TOF) que permite determinar de manera directa y simultánea los principales quelatos férricos sintéticos usados como fertilizantes, con alta selectividad y sensibilidad y buena reproducibilidad. Este método permite cuantificar los quelatos en fertilizantes comerciales y matrices agrícolas líquidas como solución del suelo, solución nutritiva, agua de riego y savia de xilema.

2. Se ha desarrollado un método de HPLC-ESI-MS(TOF) que permite la determinación de dos agentes quelantes de Fe(III), *racémico* y *meso o,o*EDDHA, en distintos tejidos (raíces, hojas, savia de xilema y fruto) y especies vegetales (tomate, remolacha y melocotonero) con muy alta selectividad y sensibilidad.

3. La elección de estándar interno ha sido un factor crítico en el desarrollo de un método para determinar estos dos agentes quelantes, *racémico* y *meso o,o*EDDHA -en forma de quelatos férricos- en tejidos vegetales por HPLC-ESI-MS. Para este caso en particular los estándares internos del quelato marcado con isótopos estables de Fe no han resultado adecuados, ya sea debido a la presencia de reacciones de intercambio isotópico o bien a la existencia de interferencias isobáricas. Un análogo estructural, el compuesto metilado del *o,o*EDDHA, ha resultado ser un buen estándar interno.

4. Se han descrito los patrones de fragmentación ESI-CID-MS^2 de la mayoría de los quelatos férricos sintéticos utilizados como fertilizantes. En general, los iones producto más intensos correspondieron a descarboxilaciones, a excepción de los quelatos Fe(III)-CDTA, Fe(III)-*o,p*EDDHA, Fe(III)-EDDCHA y Fe(III)-EDDHSA, en los que correspondieron a pérdidas en el anillo. Cuando los espectros se obtuvieron con un analizador de masas QTOF se consiguió diferenciar entre isómeros posicionales como Fe(III)-*o,o*EDDHA y Fe(III)-*o,p*EDDHA.

5. Se han propuesto dos estructuras para dos compuestos de Fe desconocidos presentes en un fertilizante comercial de Fe(III)-EDDHA con la técnica de HPLC-ESI-CID-MS^2(QTOF). Estos compuestos son quelatos férricos con una estructura similar a la del EDDHA, derivados probablemente de impurezas de los reactivos de partida en la síntesis del agente quelante.

6. El marcaje diferencial con dos isótopos estables de Fe (^{54}Fe y ^{57}Fe) de los isómeros *racémico* y *meso* Fe(III)-*o,o*EDDHA ha resultado ser una herramienta de gran utilidad para estudios de nutrición de hierro en plantas, incluyendo absorción, transporte y distribución del Fe aportado.

7. El isómero *meso* Fe(III)-*o,o*EDDHA ha resultado ser dos veces más eficiente para suministrar Fe que el isómero *racémico* Fe(III)-*o,o*EDDHA, en plantas de remolacha deficientes en Fe y crecidas en solución nutritiva. La velocidad de reducción del isómero *meso* Fe(III)-*o,o*EDDHA fue dos veces superior con respecto a la del *racémico* Fe(III)-*o,o*EDDHA.

8. Los agentes quelantes *racémico* y *meso o,o*EDDHA son rápidamente absorbidos por las plantas de remolacha deficientes en Fe, encontrándose cantidades significativas en todos los tejidos después de sólo 3 horas de tratamiento.

9. Los agentes quelantes *racémico* y *meso o,o*EDDHA son absorbidos y distribuidos con una relación 1:1 en plantas de remolacha deficientes en Fe, siendo la cantidad de los mismos un orden de magnitud menor que la de Fe tomado en todas las partes de la planta. Este hecho indica que aunque la mayoría del Fe se toma a través de un mecanismo que implica la disociación del quelato férrico, una pequeña parte puede ser tomado mediante mecanismos que no incluyen disociación.

10. Después de 6 horas de tratamiento, la mayor parte del Fe absorbido por plantas de remolacha deficientes en Fe se localizó en las raíces mientras que los agentes quelantes *racémico* y *meso o,o*EDDHA se encontraron preferentemente en las hojas.

Anexo I

PUBLICACIONES INCLUIDAS EN LA TESIS:

Los índices de impacto de las revistas corresponden al año en que se publicó el artículo. En el caso de artículos del 2009 y 2010 se ha cogido el valor del 2008.

(1) Orera I, Abadía J, Abadía A, Álvarez-Fernández A. Analytical technologies to study the biological and environmental implications of iron-fertilisation using synthetic ferric chelates: the case of Fe(III)-EDDHA. *Journal of Horticultural Science and Biotechnology* (2009) 84(1): 7-12. Área temática: Horticulture. Índice de impacto: 0.862

(2) Álvarez-Fernández A*, Orera I*, Abadía J, Abadía A. Determination of synthetic ferric chelates used as fertilizers by liquid Chromatography-Electrospray/Mass Spectrometry in agricultural matrices. *Journal of the American Society for Mass Spectrometry* (2007) 18: 37-47. Área temática: Analytical Chemistry. Índice de impacto: 3.664. *Ambos autores han contribuido igualmente a la realización de este trabajo.

(3) Orera I, Abadía A, Abadía J, Álvarez-Fernández A. Determination of *o,o*EDDHA -a xenobiotic chelating agent used in Fe-fertilizers- in plant tissues by liquid chromatography-electrospray mass spectrometry: overcoming matrix effects. *Rapid Communications in Mass Spectrometry* (2009) 23(11):1694-1702. DOI: 10.1002/rcm.4056. Área temática: Analytical Chemistry. Índice de impacto: 2.772.

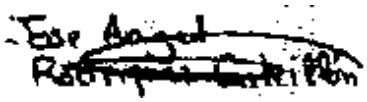
(4) Orera I, Orduna J, Abadía J, Álvarez-Fernández A. Electrospray-collision-induced dissociation mass spectrometry: a tool to characterize synthetic polyaminocarboxylate ferric chelates used as fertilizers. *Rapid Communications in Mass Spectrometry* (2010) 24(1):109-119. DOI: 10.1002/rcm.4361. Área temática: Analytical Chemistry. Índice de impacto: 2.772.

(5) Orera I, Rodríguez-Castrillón J.A., Moldovan M., García-Alonso J.I., Abadía A, Abadía J., Álvarez-Fernández A. Using a dual-stable Fe isotope tracer method to study the uptake, xylem transport and distribution of Fe and chelating agent from Fe(III)-*o,o*EDDHA stereoisomers in Fe-deficient Strategy I plants. *Metallomics* (enviado).

JOSE ÁNGEL RODRÍGUEZ CASTRILLÓN, coautor del artículo "Using a dual-stable Fe isotope tracer method to study the uptake, xylem transport and distribution of Fe and chelating agent from Fe(III)-*o,o*EDDHA stereoisomers in Fe-deficient Strategy I plants. Orera I., Rodríguez-Castrillón J.A., Moldovan M., García-Alonso J.I., Abadía A., Abadía J., Álvarez-Fernández A. *Metallomics*".

DECLARA
Que renuncia a presentar dicho artículo como derivado de su trabajo de Tesis Doctoral.

Oviedo, 22 de Octubre de 2009



Fdo. José Ángel Rodríguez- Castrillón

Anexo II

Fruto de colaboraciones en el desarrollo de esta Tesis Doctoral son también las siguientes publicaciones:

1. Fernández V, Orera I, Abadía J, Abadía A. Foliar iron-fertilisation of fruit trees: present knowledge and future perspectives – a review. *Journal of Horticultural Science and Biotechnology* (2009) 84(1): 1-6.

2. Rellán-Álvarez R, Giner-Martínez-Sierra J, Orduna J, Orera I, Rodríguez-Castrillón JA, García-Alonso JI, Abadía J, Álvarez-Fernández A Identification of a tri-iron(III), tri-citrate complex in the xylem sap of iron-deficient tomato resupplied with iron: new insights into plant iron long-distance transport. *Plant Cell Physiology* (2010) 51: 91-102.

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Foliar iron-fertilisation of fruit trees: present knowledge and future perspectives – a review

By V. FERNÁNDEZ, I. ORERA, J. ABADÍA* and A. ABADÍA
Department of Plant Nutrition, Aula Dei Experimental Station (CSIC), P.O. Box 13034, 50080 Zaragoza, Spain
(e-mail: jabadia@eead.csic.es) *(Accepted 22 July 2008)*

SUMMARY
Iron (Fe)-deficiency is a common physiological disorder affecting fruit crops in many areas of the World. Foliar Fe-fertilisation is a common agricultural strategy to control Fe-deficiency under field conditions. However, variable responses to Fe sprays have often been described and foliar Fe-fertilisation cannot yet be considered a reliable strategy to control plant Fe-deficiency. The lack of understanding of some factors relating to the penetration, distribution, and bio-activity of leaf-applied, Fe-containing solutions hinders the development of effective Fe formulations for foliar treatment. The current state-of-the-art and future perspectives for foliar Fe-fertilisation, as a strategy to control Fe-deficiency in fruit crops, is discussed.

Iron (Fe)-deficiency chlorosis is a widespread physiological disorder affecting many fruit crops and is a limiting factor for production, especially under high pH, calcareous soil conditions, such as those prevailing in many agricultural areas with a Mediterranean climate. Typical symptoms of Fe-deficiency include the development of interveinal chlorosis, starting from the apical leaves, reduction of shoot growth, defoliation during the growing season and, ultimately, tree death (Rombolà and Tagliavini, 2006). Iron chlorosis has deleterious effects on fruit production, reducing the number of fruits per tree, fruit size, total yield, and affecting fruit quality parameters such as colour, firmness, or acidity (Álvarez-Fernández *et al*., 2003; 2006).

There is scientific evidence that Fe-fertilisation increases fruit quality and yield in many crops (Álvarez-Fernández *et al*., 2006). Iron-fertilisation is a standard agricultural practice in fruit production areas that suffer from plant Fe-deficiency. Strategies to alleviate Fe-chlorosis in fruit crops include: (i) the use of rootstocks tolerant to soil conditions that induce the development of the disorder and with improved Fe-uptake mechanisms; (ii) modifying soil characteristics; and/or (iii) treatment with Fe-substances *via* root, trunk, or canopy application(s) (Abadía *et al*., 2004; Lucena, 2006). Iron-fertilisation of roots is the most reliable and widely-used technique to control Fe-deficiency, and commercial Fe(III)-EDDHA-based products are the most effective fertilisers used to correct Fe-chlorosis under severe soil conditions (Lucena, 2006). However, such chemicals are expensive and may perform differently according to the particular Fe(III)-EDDHA formulation (Cerdán *et al*., 2007).

Foliar Fe-fertilisation could be a cheaper and more targeted strategy to correct plant Fe-chlorosis (Abadía *et al*., 2002; Álvarez-Fernández *et al*., 2004; Fernández *et al*., 2008a), but the response to Fe sprays has been shown to vary according to many plant-related, environmental, and physico-chemical factors (Fernández and Ebert, 2005). Problems of reproducibility and interpretation of results from foliar and cuticular Fe-application studies have been described (Fernández and Ebert, 2005). Our current limited understanding of the factors involved in the penetration, translocation, and bio-availability of leaf-applied Fe fertilisers makes it difficult to develop effective spray formulations for agricultural purposes. At present, foliar nutrition is only considered to be a valuable complement to the application of nutrients *via* the root system (Weinbaum, 1996).

In general, the penetration of Fe-containing solutions will be influenced by plant factors, environmental conditions, the nature of the spray solution, and the method of application (Currier and Dybing, 1959). Similarly, the roles of active and passive processes involved in the penetration and subsequent physiological effects of foliar-applied nutrient solutions remain controversial (Jyung and Wittwer, 1964; Zhang and Brown, 1999).

The effectiveness of leaf-applied, Fe-containing solutions is normally assessed on the basis of their re-greening capacity, tissue Fe-absorption rate, and Fe-translocation from the site of treatment (Fernández, 2004; Fernández *et al*., 2006; 2008a). Therefore, in response to foliar treatment with a Fe-containing solution, at least three distinct key processes can be distinguished, in theory, although they are difficult to separate from one another: (i) the penetration of foliar-applied Fe through the leaf surface; (ii) the distribution of Fe from the site of application; and (iii) the active involvement of exogenous Fe in physiological processes.

An account of the state-of-the-art concerning foliar Fe-fertilisation of fruit trees and the key factors to be considered for the development of more effective Fe-containing formulations is provided in the following sections.

*Author for correspondence.

GENERAL CONSIDERATIONS REGARDING THE PENETRATION OF LEAF-APPLIED SOLUTIONS, WITH SPECIAL REFERENCE TO IRON

The ability of plant leaves to exchange water and solutes with the surrounding environment was recognised more than 300 years ago (see Franke, 1986). The penetration of leaf-applied chemicals was found to take place *via* the cuticle, through cuticular cracks and imperfections, the stomata, leaf hairs, and other specialised epidermal cells (Tukey *et al*., 1961). The significance of the stomatal route *vs.* the cuticular pathway remains unclear and is still an area of controversy (Eichert and Goldbach, 2008). The majority of studies on foliar uptake carried out over the last decades have focussed on the penetration of lipophilic and hydrophilic solutions through the cuticle (Fernández *et al*., 2006).

Aerial plant organs are covered by a continuous hydrophobic cuticle, which constitutes a protective barrier against water loss and limits the penetration of leaf-applied chemicals (Schönherr, 2006). The cuticle is a chemically heterogeneous membrane, consisting of an insoluble biopolymer matrix with waxes embedded in, and deposited on the surface (Jeffree, 2006).

Water repellency has been shown to be related to the crystalloids of epicuticular wax that cover the cuticular surface in a micro-relief (Barthlott and Neinhuis, 1997). Both a wide micro-structural diversity, and the existence of wax regeneration processes in living leaf surfaces have been reported in the last decade (Barthlott and Neinhuis, 1997; Koch *et al*., 2004). Plant cuticles are permeable to water, electrolytes, and polar compounds (Kerstiens, 2006) and the occurrence of two distinct penetration pathways has been proposed (Schönherr, 2006). Neutral, non-charged molecules may cross cuticles by dissolving and diffusing in lipophilic domains made from cutin and cuticular waxes, whereas ionic species may penetrate the leaf through aqueous pores (Schönherr, 2006), micropores, and/or spaces between molecules (Luque *et al*., 1995). The radii of cuticular aqueous pores have been estimated to range from 0.3 nm (ivy leaves) to 1.2 nm (tomato fruit; Schönherr, 2006).

In an attempt to bring together findings concerning the penetration of ionic compounds through the cuticle, Schönherr (2000; 2001) proposed the following general principles: (i) since ionic compounds in solution are surrounded by water molecules, a diffusion pathway parallel to the lipophilic pathway must occur; (ii) air relative humidity (RH) could be a key factor in determining the rate of penetration of solutions; (iii) given the importance of RH, active ingredients, either hygroscopic or with low points of deliquescence, will promote foliar penetration; (iv) concentration would serve as driving force for penetration, and the absolute amount which penetrates would probably be proportional to concentration; (v) smaller molecules should penetrate faster than larger ones; (vi) cations and anions will penetrate in equivalent amounts; and (vii) effective wetting agents can greatly increase the rate of penetration of electrolyte solutions. In contrast, stomatal uptake may resemble diffusion in bulk water, and therefore should not be restricted by the factors mentioned above (Eichert *et al*., 2006).

Iron sprays are generally based on Fe-chelates or Fe-salt solutions and can be expected to be taken up *via* the cuticle or stomata. However, recent evidence showed that foliar uptake of Fe-containing solutions does not fully concur with the seven principles described above. Fernández *et al*., (2005; 2006; 2008a) and Schönherr *et al*. (2005) observed no correlation between molecular mass and penetration rate. The Fe(III)-chelates commonly used as Fe sprays have sizes ranging from 1.0 – 1.3 nm (maximum radii) and 0.5 – 0.8 nm (minimum radii; Fernández *et al*., 2008a). Iron complexes in solution will be larger, due to hydration, but will remain smaller than stomatal apertures (e.g., 20 – 30 µm pore size for pear and peach leaves). Chelates may also penetrate the leaf through the cuticular pathway, since the sizes of Fe-compounds and the pores may be comparable.

Schönherr *et al*. (2005) and Fernández (2004) detected a negative correlation between the concentration of Fe(III)-chelates applied and the penetration rate, expressed as a percentage of the amount applied, which nevertheless represented a high amount of Fe penetrating the leaf or the cuticle. A similar effect has been described for the penetration of foliar-applied K (Chamel, 1996) and other elements (Tukey *et al*., 1961; Middleton and Sanderson, 1965) and also for 2,4-diclorophenoxyacetic acid (2,4-D; Liu, 2004). Chamel (1996) hypothesised that the decrease in relative penetration rate with higher K concentrations may have been due to progressive saturation of the sites for uptake. A similar phenomenon was observed with Fe(III)-chelates, and it was suggested that high concentrations of these compounds may reduce the size of the aqueous pores (Schönherr *et al*., 2005). A reduction in water conductance through fruit cuticles after $FeCl_3$ treatment was reported by Weichert and Knoche (2006), which may have been caused by a competition for water between Fe(III) and ionised carboxyl groups in the cuticle, leading to partial dehydration of the pore (Schönherr, 2006). However, a different mechanism could be expected for the Fe(III)-chelates used in foliar sprays, since they have high stability constants ($\log_{10} K^0$ generally ranging from 10 to 25), and most of them are negatively-charged between pH 5 and pH 7 (the normal pH range of foliar sprays). It may be possible that, under the prevailing pH, concentration, and photo-reduction conditions, the functional groups in the Fe(III)-chelates could interact with each other, forming polynuclear Fe-complexes (Rich and Morel, 1990), and also with other formulation ingredients, components of the cuticle, and/or the walls limiting the apoplastic space.

FACTORS AFFECTING THE EFFICIENCY OF IRON-CONTAINING SOLUTIONS

Environmental factors

Environmental factors such as RH, temperature, and light will play a role with regard to droplet drying, cuticular hydration, and the physiological state of the plant (Currier and Dybing, 1959), among other parameters. Under field conditions, there is a continuous interaction between such factors, which will provoke diverse physiological responses. Thus, the results from field studies on foliar fertilisation may differ from those

obtained with annual plants and/or under controlled environmental conditions.

As described above, RH could be a key factor influencing the penetration of foliar sprays. At a high RH, cuticular permeability may be increased through hydration, and the drying of salt deposits will be delayed (Currier and Dybing, 1959; Schönherr, 2001). Substances with deliquescence points below the prevailing RH will remain as solutes, and leaf penetration will continue. In a study developed using cuticular membranes of astomatous *Populus* × *canescens* and different Fe-compounds, Schönherr *et al.* (2005) reported that the cuticle penetration rate was insignificant at RH ≤ 90%. These authors raised doubts about the usefulness of Fe-sprays under the arid and semi-arid conditions found in most of the areas of the World affected by Fe-chlorosis. However, foliar Fe-fertilisation trials carried out at RHs ranging from 30 – 60% showed the beneficial effects of applying Fe-substances to Fe-deficient fruit crops such as citrus, pear, peach, apple, mango, plum, and almond, both in terms of leaf re-greening and improved fruit yield and quality (Fernández *et al.*, 2006; 2008a).

Currier and Dybing (1959) mentioned the positive effects of moderately warm temperatures in stimulating foliar penetration through changes in physiological processes (e.g., increased photosynthesis and translocation) and in the physico-chemical factors of the spray solution. Cuticular penetration of Fe-containing solutions at 100% RH did not seem to be affected by temperature changes ranging from 15° – 35°C (Schönherr *et al.*, 2005).

Light is known to stimulate stomatal opening and various physiological processes such as photosynthesis or xylem flux (Currier and Dybing, 1959; Jyung and Wittwer, 1964), which may influence foliar Fe-uptake at some stage. Since Fe is thought to be taken up into the symplast *via* a light-dependent, plasma membrane-bound Fe(III) reductase, light may stimulate a sink-to-source Fe gradient within the leaf tissue, thereby promoting Fe penetration. Light has been shown to increase the rate of Fe-uptake and the distribution of leaf-applied Fe-containing solutions (Fernández, 2004; Schlegel *et al.*, 2006; Wallihan *et al.*, 1964).

The prevailing environmental conditions will also affect leaf morphology and structure in terms of, for example, cuticle thickness or the amount and/or composition of waxes (Currier and Dybing, 1959; Koch *et al.*, 2004).

Plant-related factors

The uptake of leaf-applied chemicals can be expected to vary according to the particular plant species, variety, and growing conditions used, which will ultimately determine factors such as leaf morphology, structure, and the rates of physiological processes.

Both the upper and lower leaf surfaces are involved in the process of foliar uptake. Several investigators have reported on the penetrability of the lower epidermis *vs.* the upper epidermis, which will be governed chiefly by stomatal and cuticular variations between the two leaf surfaces (Currier and Dybing, 1959; Wòjcik, 2004).

The structure and composition of the cuticle, as well as the morphology, distribution, and sizes of the stomata and leaf hairs, differ between plant species and may influence foliar uptake processes. Variations in cuticular structure and composition will result in differences in leaf wettability, retention and penetration of substances. Selective permeability of cuticles around the cuticular edges of guard cells has often been reported (Eichert *et al.*, 2001; Schlegel *et al.*, 2006; Schönherr, 2006).

Young, partially-expanded leaves are more penetrable than fully-expanded leaves (Sargent and Blackman, 1962). The stomata present in old leaves may fail to open (Turner and Begg, 1973) or, in the case of citrus leaves, may develop "plugs" (Turrell, 1947), thereby leading to lower penetration rates compared to younger leaves.

Factors relating to the physiological state of the plant such as root temperature, root osmotic potential, or nutrient status, may also modulate the effectiveness of foliar fertilisation (Weinbaum, 1996). Regarding Fe-chlorosis in fruit trees, structural (Maldonado-Torres *et al.*, 2006) and morphological (Fernández *et al.*, 2008b) changes have been observed, as illustrated in Figure 1 A, B for Fe-sufficient and Fe–deficient adaxial leaf surfaces in cherry trees, respectively. Iron-deficient

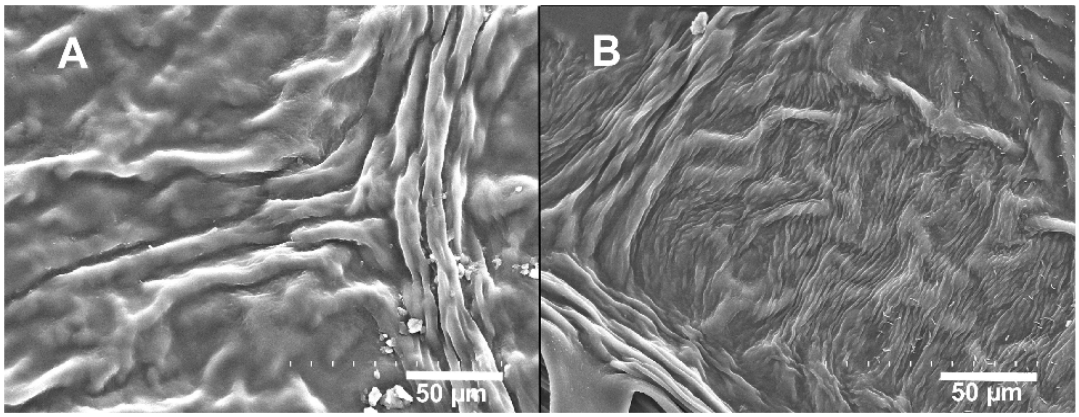

FIG. 1
Scanning electron micrographs of the adaxial leaf surface from an Fe-sufficient (green) cherry leaf (Panel A) and an Fe-deficient (chlorotic) cherry leaf (Panel B). Scale bars = 50 µm.

leaves have an altered surface appearance. Differences related to Fe-chlorosis with regard to the cuticle and waxes have been observed in peach, cherry, sugarbeet, and pear, and work is in progress to determine the significance of such variations in terms of physiological processes and the foliar uptake of Fe-fertilisers (Fernández *et al.*, 2008b).

Effect of formulation adjuvants

The possibility of improving foliar uptake of Fe-containing solutions has long been recognised (Fernández and Ebert, 2005). According to Schönherr *et al.* (2005), the addition of hygroscopic humectants may be the only option to improve the performance of Fe-sprays in arid and semi-arid areas.

Surfactants increase spray droplet retention and wetting, but their effect on the uptake of foliar sprays is complex and remains unclear (Liu, 2004). There is evidence that interactions between formulation components occur, but currently it is not possible to predict, *a priori*, the performance of a leaf-applied agrochemical in combination with a particular surfactant (Liu, 2004).

An example of the interactions between Fe-compounds and surfactants, as observed by Neumann and Prinz (1975), is illustrated in Table I. Iron concentrations in leaf tissue after foliar Fe-fertilisation were lower when using solutions of Fe(III)-EDTA in pure water than when using the same Fe compound and any of three different surfactants. Also, Surfactant 1 led to the highest tissue concentrations of Fe, suggesting the occurrence of different interactions between Fe-compounds and surfactants, at some stage (Fernández *et al.*, 2008a).

In an attempt to trace the interactions between surfactant and Fe(III)-chelate molecules, surfactant and Fe-containing mixtures were analysed by electrospray-ionisation time-of-flight mass spectrometry (ESI-TOF MS). This technique enables accurate identification and quantification of ionisable compounds by using exact mass/charge (m/z) ratio determinations (Álvarez-Fernández *et al.*, 2007). It is also an interesting tool with which to analyse surfactants because it provides qualitative information on surfactant structure concerning its molecular weight distribution and functional end-group identification.

The mass spectrum of Fe(III)-EDTA obtained with ESI-TOF MS in negative ion mode is represented in Figure 2A. The major peak corresponds to the $[M - H]^{-1}$ molecular ion at an m/z value of 344.0. The mass spectrum of a non-ionic surfactant (an organosilicon) is shown in Figure 2B. Since MS with an electrospray ionisation source detects only ionic or ionisable molecules, no signal was detected in the mass spectrum with the non-ionic surfactant. The mass spectrum of Fe(III)-EDTA supplemented with the surfactant is represented in Figure 2C. A major peak at an m/z value of 344.0, corresponding to the $[M - H]^{-1}$ molecular ion of the Fe(III)-EDTA chelate, was found once again. However, the occurrence of a negatively-charged polymer in the m/z range of 550 – 1,000 was detected. The m/z distance between monomeric units was 14, which is typical of ethylene-glycol and propylene-glycol hetero-polymers. These results show that the non-ionic

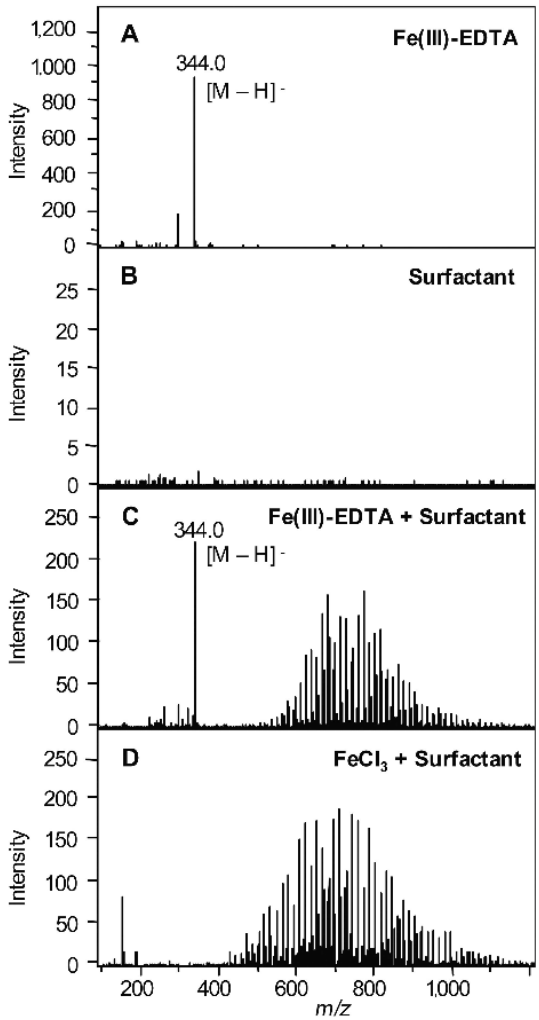

FIG. 2
Electrospray-ionisation time-of-flight (ESI-TOF) MS spectra of Fe(III)-EDTA (Panel A), Surfactant 1 (an organosilicon; Panel B); Fe(III)-EDTA plus Surfactant 1 (Panel C); and $FeCl_3$ plus Surfactant 1 (Panel D). Data were acquired in negative ion mode by injecting aqueous solutions of 50 µM Fe(III)-EDTA (Panel A), 1 g l^{-1} Surfactant 1 (Panel B), 50 µM Fe(III)-EDTA plus 1 g l^{-1} Surfactant 1 (Panel C); and 100 µM $FeCl_3$ plus 1 g l^{-1} Surfactant 1 (Panel D). The mass spectrometer was operated with end-plate and spray tip potentials of 2.8 kV and 3.3 kV respectively, an orifice voltage value of 120 V, and a drying gas temperature of 200°C.

TABLE I
Leaf tissue Fe-concentrations 1 month after foliar treatment with 2 mM Fe(III)-EDTA in combination with 1 g l^{-1} formulations of three different non-ionic surface-active agents or water (i.e., no surfactant)

Surfactant treatment	Fe concentration (mg g^{-1} DW)
Surfactant 1[†]	245.3 ± 6.1 c[‡]
Surfactant 2	173.0 ± 3.5 b
Surfactant 3	185.1 ± 4.1 b
Pure water (no surfactant)	134.0 ± 3.1 a

[‡]Mean values followed by a different lower-case letter indicate different levels of significance according to Duncan's multiple range test ($P \leq 0.05$). Data are means ± SE (n = 2; 30 leaves per sample).
[†]Surfactant 1 was an organosilicon Surfactant 2 was an ethoxylated oil, and Surfactant 3 was an alkyl polyglucoside.

surfactant was ionised due to the presence of the Fe(III)-EDTA chelate.

Usually, Fe(III)-chelates are prepared by addition of $FeCl_3$ to the corresponding chelating agent, in this case K_2EDTA. Therefore, the hypothesis that Cl^- ions may be responsible for the ionisation of the polymer was subsequently tested. The mass spectrum of $FeCl_3$ plus 0.1% (v/v) Surfactant 1 is represented in Figure 2D. An identical polymer to the one observed in Figure 2C was formed. These results suggest that the Cl^- ions present in the Fe(III)-chelate solution may induce ionisation of the surfactant. The same polymer was observed in the mass spectrum of Fe(III)-EDTA synthesised from Na_2EDTA and $FeCl_3$ with Surfactant 1 (data not shown).

In summary, ions derived from the synthesis of the Fe(III)-chelate could induce ionisation of non-ionic surfactants due to "salting out" effects (Mackay, 1997), as shown above. This could affect the performance of surfactants as adjuvants in foliar sprays. Research is in progress to understand the interactions between Fe-substances and surfactants suitable for foliar application.

CONCLUSIONS AND FUTURE PERSPECTIVES

The performance of Fe-sprays is affected by many plant-related, environmental, and physico-chemical factors, which are currently not fully understood. Research should focus on investigating the potential interactions between formulation components using modern analytical techniques such as those described above. Efforts should be made to understand the relevance of the physico-chemical properties of spray solutions to design optimised Fe-containing formulations, and the significance of changes in the leaf surface in relation to the foliar uptake of agrochemicals. The process of penetration of a leaf-applied, Fe-containing solutions is not fully understood and should be investigated further, since foliar uptake is a prerequisite for leaf-cell Fe utilisation. Research on suitable foliar treatment strategies to ensure optimal plant coverage should also proceed. Similarly, information on plant Fe metabolism will facilitate the selection of bio-active Fe-containing compounds. The role of physiological processes and environmental factors in foliar Fe uptake and distribution should also be investigated further using intact leaves and following a holistic approach.

In summary, more knowledge relating to the role of Fe in plants, and on the effects of environmental, plant physiological, or leaf morphological factors, adopting a multi-disciplinary approach, is required for the development of effective Fe-spray formulations to correct widespread Fe-deficiency in fruit trees.

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PCP
PLANT & CELL PHYSIOLOGY

Identification of a Tri-Iron(III), Tri-Citrate Complex in the Xylem Sap of Iron-Deficient Tomato Resupplied with Iron: New Insights into Plant Iron Long-Distance Transport

Rubén Rellán-Álvarez[1], Justo Giner-Martínez-Sierra[2], Jesús Orduna[3], Irene Orera[1], José Ángel Rodríguez-Castrillón[2], José Ignacio García-Alonso[2], Javier Abadía[1,*] and Ana Álvarez-Fernández[1]

[1]Department of Plant Nutrition, Aula Dei Experimental Station, CSIC, PO Box 13034, E-50080 Zaragoza, Spain
[2]Department of Physical and Analytical Chemistry, University of Oviedo, c/Julian Clavería 8, E-33006 Oviedo, Spain
[3]New Organic Materials Unit, Institute of Materials Science of Aragón, CSIC-University of Zaragoza, c/Pedro Cerbuna 12, E-50009 Zaragoza, Spain
*Corresponding author: E-mail, jabadia@eead.csic.es; Fax, +34-976716145
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Regular Paper

The identification of Fe transport forms in plant xylem sap is crucial to the understanding of long-distance Fe transport processes in plants. Previous studies have proposed that Fe may be transported as an Fe–citrate complex in plant xylem sap, but such a complex has never been detected. In this study we report the first direct and unequivocal identification of a natural Fe complex in plant xylem sap. A tri-Fe(III), tri-citrate complex (Fe_3Cit_3) was found in the xylem sap of Fe-deficient tomato (*Solanum lycopersicum* Mill. cv. 'Tres Cantos') resupplied with Fe, by using an integrated mass spectrometry approach based on exact molecular mass, isotopic signature and Fe determination and retention time. This complex has been modeled as having an oxo-bridged tri-Fe core. A second complex, a di-Fe(III), di-citrate complex was also detected in Fe–citrate standards along with Fe_3Cit_3, with the allocation of Fe between the two complexes depending on the Fe to citrate ratio. These results provide evidence for Fe–citrate complex xylem transport in plants. The consequences for the role of Fe to citrate ratio in long-distance transport of Fe in xylem are also discussed.

Keywords: Iron deficiency • Iron-citrate • Mass spectrometry • Xylem sap • Iron transport.

Abreviations: B3LYP, hybrid density functional method; DFT, density functional theory; ESI-MS, electrospray ionization-mass spectrometry; EXAFS, extended X-ray absorption fine structure; HILIC, hydrophilic interaction liquid chromatography; HPLC, high performance liquid chromatography; IDA, isotope dilution analysis; IPD, isotope pattern deconvolution; LOD, linits of detection; NA, nicotianamine; Q-ICP-MS, quadrupole-inductively coupled plasma-mass spectrometry; TOF, time of flight; XANES, X-ray absorption near edge structure; SXRF, synchroton X-ray fluorescence.

This paper is dedicated to the memory of Dr. Arthur Wallace, a pioneer in the study of plant iron nutrition.

Introduction

The mechanisms of long-distance Fe transport in plants have remained elusive until now. In the case of xylem sap, Fe is assumed to be transported as complexed forms, because free ionic forms [Fe(II) and Fe(III)] can be toxic and are also prone to undergo precipitation at the neutral or slightly acidic pH values typical of xylem sap. Increases in carboxylate concentrations in plant xylem exudates with Fe deficiency were reported in several papers published in the 1960s by Brown and co-workers. Iron was first suggested to be transported bound to malate (Tiffin and Brown 1962), but later citrate (Cit), which also increases markedly in stem exudates of many plant species when Fe-deficient (Brown 1966) and co-migrates with Fe during paper electrophoresis (Tiffin 1966a, Tiffin 1966b, Tiffin 1970, Clark et al. 1973), was considered the most likely candidate for Fe transport.

The identity of Fe–Cit complexes in the xylem sap has only been hypothesized by means of in silico calculations using total concentrations of possible Fe complexing agents (including carboxylates) and Fe, and the known stability constants of Fe-containing complexes, always assuming that chemical equilibrium was achieved. Using this approach, several Fe–Cit species were predicted to be the most abundant Fe complexes in the xylem sap whereas other potential plant metal chelators such as nicotianamine (NA) were ruled out (von Wirén et al. 1999, Rellán-Álvarez et al. 2008) as possible xylem Fe carriers. NA function as an Fe chelator might be restricted to the cytoplasm and in Fe phloem loading (Curie et al. 2008). Citrate recently has been found by using molecular biology techniques to play a role in long-distance Fe transport. Xylem sap loading

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of Cit is seriously disrupted in two mutants, AtFRD3 (Durrett et al. 2007) and OsFRDL1 (Yokosho et al. 2009). These mutants display increased Fe-deficiency symptoms that have been associated with decreased efficiency of Fe translocation into the root vasculature.

Fe–Cit chemistry in aqueous solutions is very complex and a large number of chemical species may occur, depending on many factors (Spiro et al. 1967a, Spiro et al. 1967b, Pierre and Gautier-Luneau 2000, Gautier-Luneau et al. 2005). Direct proof of the presence of Fe–Cit complexes in xylem sap has not been obtained so far. Difficulties in detecting Fe–Cit species in xylem sap may arise for different reasons. First, both pH and Fe:Cit ratio values are known to affect markedly Fe–Cit speciation in standard aqueous solutions (Gautier-Luneau et al. 2005). Therefore, when analysis is carried out at pH values too acidic or basic (e.g. during HPLC), the speciation of Fe–Cit complexes could change and species occurring in the original xylem sample may no longer be present after chromatography. Second, analytical techniques such as mass spectrometry (MS), which have been used successfully to identify other metal complexes in the xylem sap (Ouerdane et al. 2006, Xuan et al. 2006), usually involve ionization steps (with high temperatures and voltages) that may be too harsh for relatively labile compounds such as Fe–Cit complexes. Furthermore, in most plant species the total Fe concentration in the xylem sap is in the μM range, and consequently the concentrations of the possible Fe–Cit complexes are expected to be very low.

Molecular biology approaches have provided a breadth of information about metal, metal chelator and metal complex transporters (Briat et al. 2007, Kim and Guerinot 2007, Palmer and Guerinot 2009). However, the elucidation of the molecular identity of metal complexes in plant compartments is still one of the biggest challenges in plant metal transport (Hider et al. 2004). The molecular identification of metal complexes has been tackled by two different types of technique. First, the use of highly selective and sensitive molecular and metal-specific techniques such as integrated MS (Meija et al. 2006) has been used (Ouerdane et al. 2006, Xuan et al. 2006), especially in plant fluids (e.g. xylem or phloem) where direct analysis can be carried out. Second, X-ray absorption spectroscopy, extended X-ray absorption fine structure (EXAFS) and X-ray absorption near edge structure (XANES) (Sarret et al. 2002, Küpper et al. 2004) and synchrotron X-ray fluorescence (SXRF) (Punshon et al. 2009) techniques have been applied to study metal speciation in some plant materials. The combination of metal complex elucidation techniques and molecular biology approaches should give a better picture of plant metal transport.

In this study we have used HPLC coupled to electrospray time of flight mass spectrometry (HPLC–ESI-TOFMS) and inductively coupled plasma mass spectrometry (HPLC–ICP-MS) to detect naturally occurring Fe complexes in xylem sap. Analysis conditions were kept as conservative as possible in order to maintain unaltered natural Fe species occurring in the xylem sap. With this approach we have succesfully identified a tri-Fe(III), tri-citrate complex (Fe_3Cit_3) in the xylem sap of Fe-deficient tomato plants after short-term Fe resupply. This complex has been modeled as an oxo-bridged tri-Fe(III) tri-Cit complex. A second Fe–Cit complex, the binuclear Fe(III)–Cit species Fe_2Cit_2, was only detected in Fe–Cit standard solutions along with the Fe_3Cit_3 complex, with the balance between the two complexes depending on the Fe:Cit ratio.

Results

Analysis of Fe–Cit standard solutions by integrated MS

A method to separate and identify Fe–Cit complexes was developed by analyzing Fe–Cit solutions with Fe and Cit concentrations, Fe:Cit ratios and pH values (5.5) typical of xylem sap, using HPLC and integrated MS (ESI-TOFMS and ICP-MS). In all experiments, ESI-TOFMS spectra were searched for any molecular ion having the characteristic Fe isotopic signatures, including molecular ions previously detected by ESI-MS in high concentration Fe–Cit solutions (100 mM Fe:1 M Cit) (Gautier-Luneau et al. 2005). The four different Fe stable isotopes were determined in the HPLC–ICP-MS runs. The method to determine Fe–Cit complexes was designed by optimizing first the electrospray ionization conditions, and then developing appropriate HPLC separation conditions. Throughout this study we used ^{54}Fe, ^{57}Fe and ^{nat}Fe, the latter being Fe with the natural isotopic composition: 5.85, 91.75, 2.12 and 0.28% of ^{54}Fe, ^{56}Fe, ^{57}Fe and ^{58}Fe, respectively.

Electrospray ionization conditions for Fe–Cit complexes were optimized to avoid in-source fragmentation using direct injection of a 1:10 ^{nat}Fe–Cit solution (100 μM ^{nat}Fe). Optimal ESI values for capillary exit, skimmer 1 and hexapole RF voltages were −57.1, −39.1 and 145.2 V, respectively. These values correspond to softer ESI conditions than those usually applied to low molecular weight analytes (in the 100–600 m/z range). In all conditions tested, citrate gave a strong signal at the $[CitH]^-$ mass-to-charge ratio (m/z) of 191.0 (**Supplementary Fig. 1A, B**; see inset for isotopic signature). With these soft conditions, a 10-fold increase in ionization efficiency was achieved for a m/z 366.4 signal that showed the characteristic isotopic signature of a double charged, three Fe atom-containing molecular ion. This signal can be assigned to the $[Fe(III)_3Cit_3H]^{2-}$ molecular ion (**Supplementary Fig. 1A, B**; see inset for isotopic signature). Furthermore, a second molecular ion with the characteristic isotopic signature of a double charged, three Fe atom-containing molecular ion was detected at m/z 375.4 (**Supplementary Fig. 1B**; see inset for isotopic signature). This signal can be assigned to the $[Fe(III)_3OCit_3H_3]^{2-}$ ion. The 9 m/z difference from $[Fe(III)_3Cit_3H]^{2-}$ was assigned to correspond to a labile ligand such as aquo OH_2, hydroxo OH^- or oxo O^{2-} (Gautier-Luneau et al. 2005). Both Fe–Cit molecular ions were previously reported to occur at neutral pH values and at similar Fe:Cit ratios (Gautier-Luneau et al. 2005). The relative intensities of the different peaks did not match those found by Gautier-Luneau et al. (2005), probably due to differences

in MS devices and solution pH values (at least one pH unit of difference). Conversely, signals at m/z 300.4 and 271.9 appeared under standard ESI conditions (**Supplementary Fig. 1A**) but they were significantly reduced after ESI optimization (**Supplementary Fig. 1B**), suggesting that they may correspond to in-source fragmentation products (mainly due to decarboxylation processes, very common in this kind of compound) of the Fe_3Cit_3 produced at higher voltage values.

HPLC separation conditions were also optimized to obtain a good separation of the Fe–Cit complexes from other matrix components (e.g. Cit) that could interfere in the ESI process. The range of HPLC options available was limited, since: (i) both Cit and Fe–Cit complexes are compounds with a high polarity; (ii) it is mandatory to maintain xylem sap-typical pH values during chromatography; (iii) the method must be suitable for ESI-TOFMS and ICP-MS detection. Different approaches, including several column types, elution programs and eluents—acetonitrile and methanol—were tested. The best results were obtained with a zwitterionic hydrophilic interaction column (ZIC-HILIC; Sequant), previously used to separate polar Fe compounds such as Fe(II)–NA, Fe(III)–deoxymugineic acid [Fe(III)–DMA] and others (Xuan et al. 2006). Two parallel systems, an HPLC–ICP-MS and an HPLC–ESI-TOFMS, were used to gain knowledge about atomic and molecular identity, respectively. Experiments were carried out with ^{54}Fe–Cit and also with ^{nat}Fe–Cit.

HPLC–ICP-MS experiments were carried out using ^{54}Fe–Cit solutions (100 μM ^{54}Fe:1 mM Cit) to avoid ^{nat}Fe background from the HPLC system, which could be significant when determining very low concentrations of Fe by ICP-MS. Iron-54 molar flow chromatograms showed only two well-defined Fe peaks at 32.1 and 38.3 min (**Fig. 1A**) that were also observed using UV detection (**Supplementary Fig. 2A**). Using ESI-TOFMS detection, only four molecular ions with characteristic ^{54}Fe isotopic signatures (see below for identification) were found in the chromatogram, two of them at 27.1 min (241.9 and 484.9 m/z) and two more at 32.9 min (363.4 and 372.4 m/z) (**Fig. 1B**), and a UV signal was also found for these peaks (**Supplementary Fig. 2B**). The 5-min differences in retention time between HPLC–ESI-TOFMS and HPLC–ICP-MS analyses are due to the different HPLC devices used (Waters and Agilent, respectively), as judged by the shift in UV detection traces (**Supplementary Fig. 2**). A 5 mM Cit solution was also injected

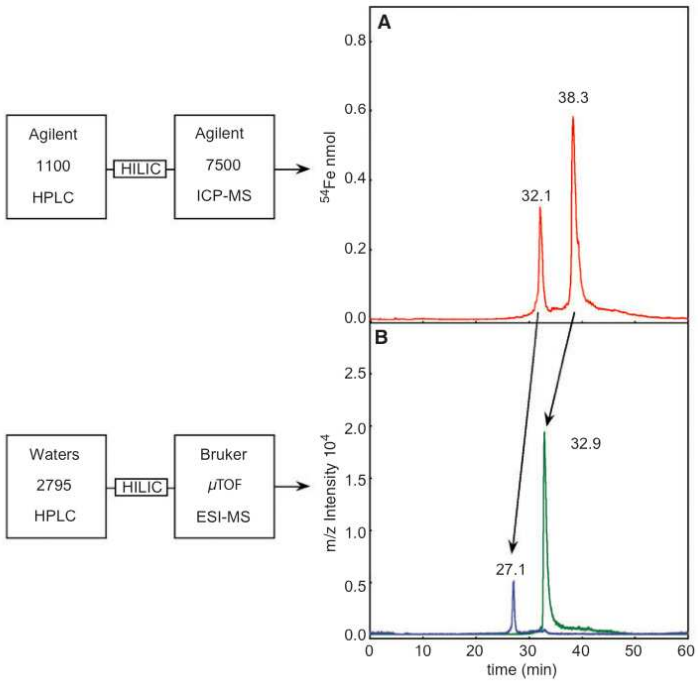

Fig. 1 HPLC–ICP-MS (A) and HPLC–ESI-TOFMS (B) chromatograms of a ^{54}Fe–Cit standard solution (Fe:Cit ratio 1:10, 100 μM ^{54}Fe, pH 5.5, in 50% mobile phase B) showing peaks corresponding to Fe complexes. HPLC–ESI-TOFMS traces (B) are the sum of molecular ions at m/z values 241.93 and 484.87 (±0.05; blue line) and 363.40 and 372.40 (±0.05; green line).

and no Fe–Cit complexes were found in the HPLC–ESI-TOFMS chromatogram, indicating that complexes found were not formed de novo during the chromatographic run.

With this HPLC method a complete separation of Fe–Cit complexes from Cit was reached, since Cit eluted as a broad peak at 5–9 min (m/z trace at 191.1, not included in **Fig. 1B**; **Supplementary Fig. 3A**). Other Fe complexes putatively occurring in plant tissues, such as Fe(III)–NA and Fe(III)–DMA, elute at retention times lower than 20 min (**Supplementary Fig. 3B, C**).

Identification of an Fe_2Cit_2 complex in Fe–citrate standard solutions

When using ^{54}Fe–Cit, the Fe peak at 27.1 min in HPLC–ESI-TOFMS, showed signals at m/z 241.9 and 484.9 (**Fig. 1B**) with Fe isotopic signatures characteristic of two ^{54}Fe atom-containing molecular ions, the first double charged (**Fig. 2A, Table 1**) and the second single charged (**Supplementary Fig. 4A**). Based on these exact mass and isotopic pattern data, the Sigma Fit algorithm (Ojanperä et al. 2006) proposed $^{54}Fe_2C_{12}H_8O_{14}$ and $^{54}Fe_2C_{12}H_9O_{14}$ as the most accurate formulae, corresponding to the two Fe, two Cit molecular ions $[^{54}Fe(III)_2Cit_2]^{2-}$ and $[^{54}Fe(III)_2Cit_2H]^-$, respectively (**Table 1**); these molecular ions were previously found in concentrated Fe–Cit standards (Gautier-Luneau et al. 2005). The fit of the experimental and theoretical isotopic signatures of the $[^{54}Fe(III)_2Cit_2]^{2-}$ molecular ion at m/z 241.9 is shown in **Fig. 2A** and **B**, respectively. A good fit was also found for the ion $[^{54}Fe(III)_2Cit_2H]^-$ at m/z 484.9 (**Supplementary Fig. 4A, B**).

Further confirmation of the molecular identity of the Fe_2Cit_2 complex was obtained by analyzing ^{nat}Fe–Cit standard solutions by HPLC–ESI-TOFMS, taking advantage of the characteristic ^{nat}Fe isotopic signature. The Fe peak at 27.1 min in HPLC–ESI-TOFMS showed signals at m/z 243.9 and 488.9, characteristic of two ^{nat}Fe atom-containing molecular ions, the first double charged and the second single charged (**Fig. 2C, Supplementary Fig. 4C, Table 1**). The differences in m/z values found using ^{54}Fe and ^{nat}Fe (92% ^{56}Fe) were those expected for two Fe atom-containing molecular ions (2 and 4 m/z difference for a double and a single charged ion, respectively). The Sigma Fit algorithm (Ojanperä et al. 2006) proposed $^{nat}Fe_2C_{12}H_{18}O_{14}$ and $^{nat}Fe_2C_{12}H_9O_{14}$ as the most accurate formulae corresponding to the molecular ions $[^{nat}Fe_2Cit_2]^{2-}$ and $[^{nat}Fe_2Cit_2H]^-$ (**Table 1**). The fit of the experimental and theoretical isotopic signatures of the $[^{nat}Fe_2Cit_2]^{2-}$ molecular ion (for the ^{56}Fe signal at m/z 243.9) is shown in **Fig. 2C** and **D**, respectively. A good fit was also found for the molecular ion $[^{nat}Fe_2Cit_2H]^-$ (for the ^{56}Fe signal at 488.9 m/z; **Supplementary Fig. 4C, D**).

Identification of an Fe_3Cit_3 complex in Fe–citrate standard solutions

The Fe peak at 32.9 min in HPLC–ESI-TOFMS analysis of ^{54}Fe–Cit solutions (**Fig. 1B**) showed signals at m/z 372.4 and 363.4, with isotopic signatures characteristic of three ^{54}Fe atom-containing

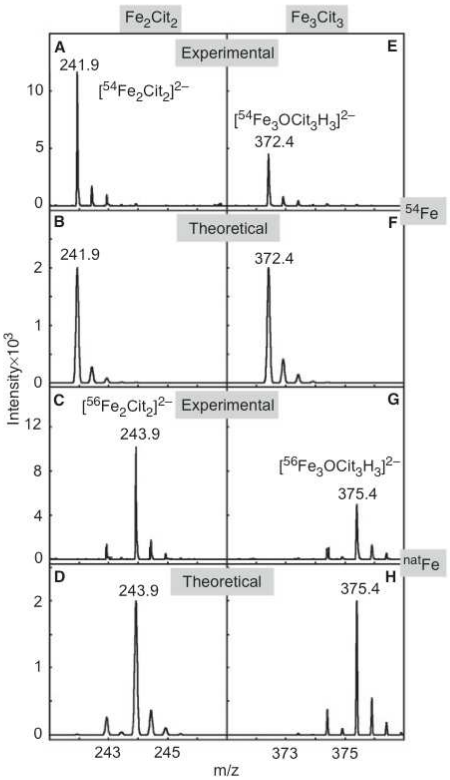

Fig. 2 Experimental (A, C, E, G) and theoretical (B, D, F, H) isotopic signatures of the molecular ions associated with Fe_2Cit_2 and Fe_3Cit_3, $[Fe_2Cit_2]^{2-}$ and $[Fe_3OCit_3H_3]^{2-}$, respectively. Experimental data are zoomed ESI-TOF mass spectra of the Fe_2Cit_2 and Fe_3Cit_3 chromatographic peaks found when using ^{54}Fe (A, E) and ^{nat}Fe (C, G).

molecular ions, both of them double charged (**Fig. 2E, Supplementary Fig. 4E, Table 1**). The algorithm proposed $^{54}Fe_3C_{18}H_{15}O_{22}$ and $^{54}Fe_3C_{18}H_{13}O_{21}$ as the most accurate formulae, corresponding to the three Fe-, three Cit- molecular ions $[^{54}Fe_3OCit_3H_3]^{2-}$ and $[^{54}Fe_3Cit_3H]^{2-}$ (**Table 1**). The fit of the experimental and theoretical isotopic signatures of the $[^{54}Fe_3OCit_3H_3]^{2-}$ molecular ion at m/z 372.4 is shown in **Fig. 2E** and **F**, respectively. The same occurs with the ion $[^{54}Fe_3Cit_3H]^{2-}$ at m/z 363.4 (**Supplementary Fig. 4E, F**).

Further confirmation of the molecular identity of the Fe_3Cit_3 complex was obtained by analyzing ^{nat}Fe–Cit standard solutions. The peak at 32.9 min shows signals at m/z 375.4 and 366.4, characteristic of three ^{nat}Fe atom-containing molecular ions (**Fig. 2G, Table 1**); these values were 3 m/z higher than those found using ^{54}Fe. The algorithm proposed $^{nat}Fe_3C_{18}H_{15}O_{22}$ and

Table 1 Experimental ESI-TOFMS molecular ion data and parameters used to identify the molecular formulae of the Fe–Cit complexes in standard solutions and tomato xylem sap

Measured m/z	$^{56}Fe/^{54}Fe$ (Fe atoms)[a]	Charge[b]	Molecular formula	Calculated m/z	Error m/z (ppm)	SigmaFit value	Molecular ion
			Standard solutions				
241.9362	–	−2	$^{54}Fe_2C_{12}H_8O_{14}$	241.9359	2.1	0.0234	$[^{54}Fe_2Cit_2]^{2-}$
484.8781	–	−1	$^{54}Fe_2C_{12}H_9O_{14}$	484.8790	1.8	0.0157	$[^{54}Fe_2Cit_2H]^{-}$
372.4119	–	−2	$^{54}Fe_3C_{18}H_{15}O_{22}$	372.4127	2.3	0.0273	$[^{54}Fe_3OCit_3H_3]^{2-}$
363.4068	–	−2	$^{54}Fe_3C_{18}H_{13}O_{21}$	363.4074	1.4	0.0106	$[^{54}Fe_3Cit_3H]^{2-}$
243.9311	7.4 (2)	−2	$^{nat}Fe_2C_{12}H_8O_{14}$	243.9311	1.7	0.0165	$[^{nat}Fe_2Cit_2]^{2-}$
488.8705	7.1 (2)	−1	$^{nat}Fe_2C_{12}H_9O_{14}$	488.8696	2.6	0.0214	$[^{nat}Fe_2Cit_2H]^{-}$
375.4047	5.2 (3)	−2	$^{nat}Fe_3C_{18}H_{15}O_{22}$	375.4057	1.7	0.0284	$[^{nat}Fe_3OCit_3H_3]^{2-}$
366.3994	5.2 (3)	−2	$^{nat}Fe_3C_{18}H_{13}O_{21}$	366.4004	2.0	0.0294	$[^{nat}Fe_3Cit_3H]^{2-}$
			Xylem sap				
372.4097	–	−2	$^{54}Fe_3C_{18}H_{15}O_{22}$	372.4057	8.2	0.0283	$[^{54}Fe_3OCit_3H_3]^{2-}$
375.4045	5.56 (3)	−2	$^{nat}Fe_3C_{18}H_{15}O_{22}$	375.4057	6.8	0.0197	$[^{nat}Fe_3OCit_3H_3]^{2-}$

The parameters used to assess the accuracy of molecular formulae were exact molecular mass and isotopic signatures, with exact mass errors of <10 ppm, and a SigmaFit value of <0.03 (Ojanperä et al. 2006) (see Materials and Methods). Data are means of at least three independent HPLC runs.

[a]The $^{56}Fe/^{54}Fe$ ratio was used to determine the number of Fe atoms in the molecule. Theoretical $^{56}Fe/^{54}Fe$ ratios for molecules containing two or three Fe atoms are 7.9 and 5.3, respectively.

[b]The ion charge was determined by the m/z isotope difference within a given molecule. Differences of 1.0 and 0.5 m/z indicate molecular ion charges of 1 and 2, respectively.

$^{nat}Fe_3C_{18}H_{13}O_{21}$ and as the most accurate formulae, corresponding to the three Fe-, three Cit- molecular ions $[^{nat}Fe_3OCit_3H_3]^{2-}$ and $[^{nat}Fe_3Cit_3H]^{2-}$ (**Table 1**). The fit of the experimental and theoretical isotopic signatures of the $[^{nat}Fe_3OCit_3H_3]^{2-}$ molecular ion (for the ^{56}Fe signal at m/z 375.4) is shown in **Fig. 2G** and **H**, respectively. A good fit was also found for the ion $[^{nat}Fe_3Cit_3H]^{2-}$ (for the ^{56}Fe signal at 366.4 m/z; **Supplementary Fig. 4G, H**).

Iron to citrate ratios drive the balance between Fe_2Cit_2 and Fe_3Cit_3 in standard solutions

To assess the influence of the Fe:Cit ratio on the balance of Fe–Cit complexes, standard solutions with Fe:Cit ratios of 1:1, 1:10, 1:100 and 1:500 (at 100 μM Fe) were analyzed by HPLC–ESI-TOFMS and HPLC–ICP-MS, always at the pH value typical of xylem sap. Both detection systems show that high Fe:Cit ratios favor the formation of Fe_3Cit_3, whereas lower Fe:Cit ratios lead to the formation of Fe_2Cit_2 (**Fig. 3**). With Fe:Cit ratios >1:10, Fe_3Cit_3 would account for >75% of the total complexed Fe, whereas with Fe:Cit ratios <1:75, Fe_2Cit_2 would account for >75% of the total. In the range between these ratios both complexes would be present. In all cases, no other Fe-containing peaks different from Fe_2Cit_2 and Fe_3Cit_3 were found by HPLC–ICP-MS or HPLC–ESI-TOFMS (**Supplementary Fig. 5**).

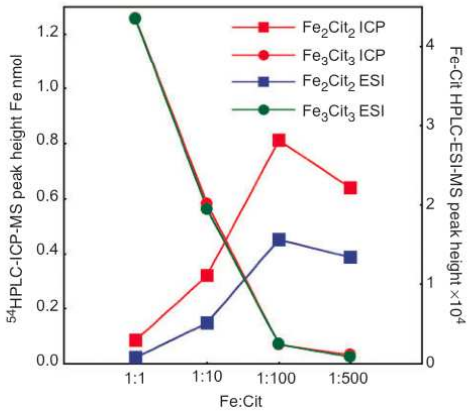

Fig. 3 Effect of the Fe:Cit ratio on the Fe_2Cit_2 and Fe_3Cit_3 balance. Data are chromatographic peak maximum heights obtained with ICP-MS and ESI-TOFMS detection. ^{54}Fe–Cit standard solutions with Fe:Cit ratios of 1:1, 1:10, 1:100 and 1:500 (at 100 μM ^{54}Fe, pH 5.5 in 50% mobile phase B) were used.

Quantification of Fe–Cit complexes in Fe–citrate standard solutions

We attempted to quantify the amount of Fe associated with the Fe–Cit complexes found in ^{54}Fe–Cit solutions by using HPLC–ICP-MS ^{54}Fe molar flow chromatograms. The sum of the Fe–Cit complexes Fe_2Cit_2 and Fe_3Cit_3 accounted for approximately 60% ($n = 4$) of the total injected Fe. However, only 67% of the Fe was eluted from the HPLC. Therefore, the Fe contained in the Fe_2Cit_2 and Fe_3Cit_3 peaks accounted for 91% of the eluted Fe. When the analysis was carried out with longer run times

Table 2 Xylem sap pH, Fe and citrate concentrations, Fe:Cit ratios and Fe_3Cit_3 and Fe_2Cit_2 complexes (in % of the chromatographically eluted Fe), in Fe-sufficient (+Fe), Fe-deficient (−Fe) and Fe-deficient tomato plants resupplied with Fe–*o,o*EDDHA for 12 h (Fe-resupplied)

Fe status	Fe in solution (μM)	pH	Fe (μM)	Cit (μM)	Fe:Cit	Fe_3Cit_3 (%)	Fe_2Cit_2 (%)
+Fe	45	5.8±0.1	19.9±2.6	11.6±6.6	1:0.6	bld[a]	bld
−Fe	$0+HCO_3^-$	5.8±0.1	5.4±4.4	165±9.2	1:31	bld	bld
Fe-resupplied	$(0+HCO_3^-)+45$	5.5±0.1	121.0±13.7	172.2±12.6	1:1.4	71[b]	bld

Data are means±SE of at least three independent samples.
[a]bld: below detection limit.
[b]Since only 25% of the total injected Fe was eluted from the column, this percentage corresponds to 16% of the total injected Fe.

a broad Fe peak eluted at approximately 70 min, indicating that at least an additional Fe form either occurred in the original sample or was formed from Fe–Cit complexes during the LC run. The UV–visible spectra of this peak suggest that it may correspond to Fe-oxyhydroxides.

The Fe_3Cit_3 complex is present in the xylem sap of Fe-deficient tomato plants resupplied with Fe

We analyzed xylem sap to look for the presence of possible Fe–Cit complexes by the optimized methodology described above. We used xylem of Fe-sufficient, Fe-deficient and Fe-deficient plants resupplied with Fe–ethylenediamine-*N*-*N*′ bis(*o*-hydroxyphenylacetic) acid (*o,o*EDDHA) for 6, 12 and 24 h, where Fe concentrations were 19.9±2.6, 5.4±4.4, 42.9±3.7, 121.0±13.7 and 43.5±9.1 μM ($n=4$), respectively (**Table 2**). The xylem sap of plants resupplied for 12 h was chosen for further studies, since they have the highest concentrations of Fe. These xylem sap samples showed a major ^{54}Fe peak in HPLC–ICP-MS at 39.5 min (**Fig. 4A**). When using HPLC–ESI-TOFMS, an Fe peak eluted at 34.1 min and showed signals at m/z 372.4 or 375.4 when the Fe source was ^{54}Fe-*o,o*EDDHA or ^{nat}Fe-*o,o*EDDHA, respectively (**Fig. 4B, C**). The retention time difference between HPLC–ICP-MS and HPLC–ESI-TOFMS was due to the use of different HPLC systems as explained above. Based on this exact mass and the isotopic pattern data (see insets of **Fig. 4B, C**), the algorithm proposed $Fe_3C_{18}H_{15}O_{22}$ as the more accurate molecular formula (**Table 1**; ^{56}Fe signal m/z error 6.8 ppm and SigmaFit value 0.0197), corresponding to the Fe_3Cit_3 molecular ion $[Fe_3O(Cit)_3H_3]^{2-}$ also found in the Fe–Cit standards (**Figs. 1, 2**). The approximately 1-min difference in retention time for Fe_3Cit_3 between xylem sap samples (**Fig. 4**) and standards (**Fig. 1**) in both HPLC systems was likely due to matrix effects. It should be noted that although the Fe_3Cit_3 complex was detected in standard solution as a mixture of two ions $[Fe_3Cit_3H]^{2-}$ and $[Fe_3OCit_3H_3]^{2-}$, in xylem sap only the latter was found. No other Fe-containing molecular ions, including Fe–NA, were found in the whole HPLC–ESI-TOFMS run using these HPLC conditions. However, using an HPLC–ESI-TOFMS method designed for Fe-*o,o*EDDHA analysis (Orera et al. 2009) a very low concentration of this Fe chelate (0.3±0.1 μM; $n=4$) was found. The Fe_3Cit_3 complex was also found in xylem sap of Fe-deficient plants resupplied with Fe-EDTA for 12 h, as well as in that of plants resupplied

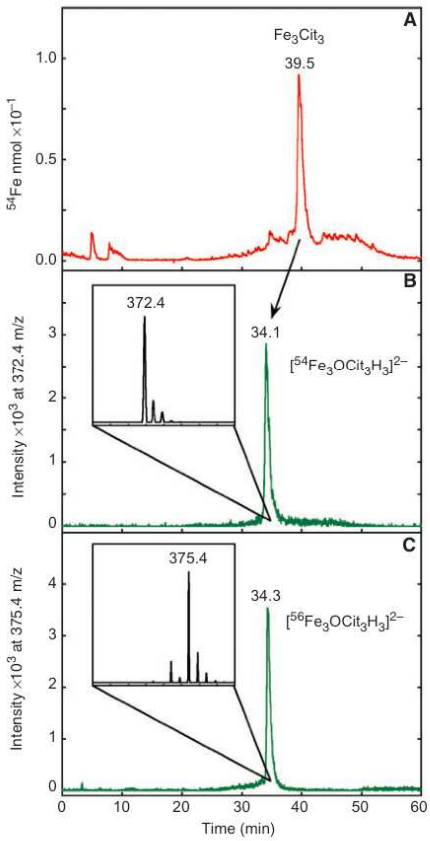

Fig. 4 HPLC–ICP-MS (A) and HPLC–ESI-TOFMS (B, C) typical chromatograms of xylem sap samples from Fe-deficient, 12-h Fe-resupplied tomato plants, showing the peak corresponding to the Fe_3Cit_3 complex. Plants were resupplied with ^{54}Fe–*o,o*EDDHA (A, B) or ^{nat}Fe–*o,o*EDDHA (C). HPLC–ESI-TOFMS traces were extracted at m/z values 372.40 and 375.40 (±0.05), corresponding to $[^{54}Fe_3OCit_3H_3]^{2-}$ and $[^{56}Fe_3OCit_3H_3]^{2-}$. Isotopic signatures of both molecular ions are shown in insets in (B) and (C).

with Fe-*o,o*EDDHA for 6 and 24 h (data not shown). However, no Fe-containing compounds could be detected in the xylem of Fe-deficient and Fe-sufficient plants.

We also attempted to quantify the amount of Fe associated with the Fe_3Cit_3 complex found in the xylem of the ^{54}Fe–*o,o*EDDHA 12 h-resupplied plants by using HPLC–ICP-MS ^{54}Fe molar flow chromatograms ($n = 3$). In these xylem samples >95% of the total Fe was ^{54}Fe. Only 25% of the injected Fe eluted from the column. The $^{54}Fe_3Cit_3$ peak accounted for the 71% of the eluted Fe and 16% of the injected Fe (**Table 2**).

Method sensitivity for the Fe_3Cit_3 complex

The sensitivity of the HPLC–ESI-TOFMS and HPLC–ICP-MS methods for the detection of the Fe_3Cit_3 complex in xylem sap can be estimated from the signal to noise ratios (s/n). Limits of detection and quantification are usually considered as the analyte concentrations giving s/n of 3 and 10, respectively. S/n found in xylem sap samples with total Fe concentrations of approximately 40 µM were 12 and 6 in HPLC–ESI-TOFMS and HPLC–ICP-MS, respectively. Therefore, in xylem sap with Fe:Cit ratios favouring the formation of Fe_3Cit_3 (>1:10; **Fig. 3**), an Fe concentration of approximately 25–30 µM will be needed for the Fe_3Cit_3 complex to be detected.

Molecular modeling of the Fe_3Cit_3 complex: an oxo-bridged tri-Fe(III) core complex

Molecular modeling of the Fe_3Cit_3 complex was carried out using the information gained from the molecular identification in xylem sap samples of Fe-deficient plants resupplied with Fe and on the basis of the known basic structure of Fe(III) carboxylates (Lippard 1988). The complex Fe_3Cit_3 has a molecular mass of 750.83 Da, a molecular formula of $Fe_3C_{18}H_{15}O_{22}$, is composed of three Cit molecules, three Fe atoms and one O atom and has two negative charges. The complex was modeled using Density Functional Theory (DFT), which has become the standard method for quantum chemical modeling of transition metals including those of biological relevance (Deeth et al. 2009 and references therein), as a trinuclear Fe(III) oxo-bridged complex (**Fig. 5, Supplementary Fig. 5**). The three Fe atoms form an equilateral triangle with an O atom in the center bridging all of them. All Fe atoms have a slightly distorted octahedral configuration. In each of the three Cit molecules, both distal carboxylate groups are bound to two Fe atoms. The four O atoms of the Cit distal carboxylate groups (two from each Cit molecule) are in the same plane of the Fe atom they complex. The two remaining positions of the Fe atom are occupied by the central carboxylate group of a Cit molecule and the O of the oxo-bridged 3-Fe center. This compact molecular geometry is further stabilized by the formation of hydrogen bonds between the hydroxyl groups and the free O atoms in the central carboxylates. Until now, six Fe–Cit complexes (three mononuclear and two dinuclear found in concentrated standard solutions, plus a nonanuclear one) have been isolated and structurally characterized in solid state (Gautier-Luneau et al. 2005 and references therein), and none of them has a central μ_3 oxygen coordinated to three Fe atoms. The Fe_2Cit_2 complex found only in Fe–Cit standard solutions was also modeled (see **Supplementary Figs. 7, 8, Supplementary Table 3**) according to the known structural characteristics found in solid state by Gautier-Luneau et al. (2005) and references therein.

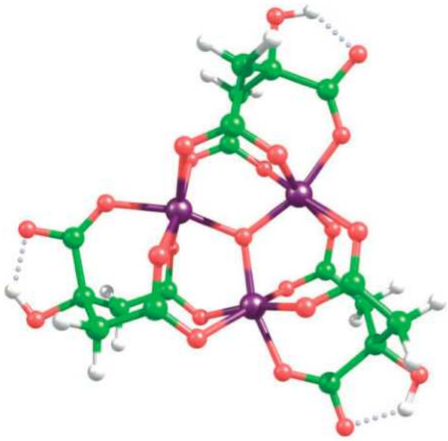

Fig. 5 Proposed structure for the Fe_3Cit_3 found in plant xylem as an oxo-bridged tri-iron–citrate complex. Iron, oxygen, carbon and hydrogen atoms are shown in purple, red, green and white, respectively.

Discussion

We report here the first direct and unequivocal identification of a natural Fe complex in plant xylem sap, Fe_3Cit_3. The complex was modeled as having an oxo-bridged tri-Fe(III) core. This is the first time that an Fe–Cit complex has been identified in biological systems. The Fe_3Cit_3 complex was identified using an integrated MS approach, based on exact molecular mass, isotopic signature, Fe content and retention time. This complex was not predicted to occur in previous in silico xylem speciation studies (López-Millán et al. 2000, López-Millán et al. 2001). A second Fe–Cit complex, Fe_2Cit_2, was also found along with Fe_3Cit_3 in standard solutions at xylem-typical pH values, with their respective abundances being tuned by the Fe:Cit ratio. The detection of Fe_3Cit_3 in the xylem sap was made possible by the use of: (i) pH values similar to those of the xylem sap throughout the analysis, (ii) a zwitterionic hydrophilic interaction column that allows for the separation of Cit and Fe–Cit complexes, (iii) high-resolution detection techniques such as ICP-MS and ESI-TOFMS, and (iv) stable Fe isotopes for identification and quantification purposes.

The changes in Fe:Cit ratios in standard solutions drive the balance between the Fe_2Cit_2 and Fe_3Cit_3 complexes, with ratios of >1:10 favoring the formation of Fe_3Cit_3 and ratios of <1:75

favoring the formation of Fe_2Cit_2 (**Fig. 3**). In the xylem sap of Fe-deficient tomato plants resupplied with Fe for 12 h, where the Fe:Cit ratio was approximately 1:1 (**Table 2**), the only complex observed was Fe_3Cit_3. The xylem sap Fe:Cit ratios in Fe-sufficient and Fe-deficient plants were approximately 1:1 and 1:30, and therefore the expected Fe–Cit complexes would be Fe_3Cit_3 and a mixture of the Fe_2Cit_2 and Fe_3Cit_3 complexes, respectively. However, no Fe–Cit complexes could be detected by HPLC–ESI-TOFMS or HPLC–ICP-MS in the xylem of these plants, likely because concentrations were below the limit of detection, estimated as approximately 25–30 μM Fe–Cit. In fact, xylem total Fe concentrations were approximately 20 and 5 μM in Fe-sufficient and Fe-deficient plants, respectively, much lower than the 43–121 μM found in Fe-deficient plants resupplied with Fe where the complex was detected. Limits of detection (LODs) for these Fe–Cit complexes are considerably higher than that reported also in xylem sap and with similar analytical techniques for the synthetic Fe chelate Fe(III)–*o,o*EDDHA, which is <1 μM (Orera et al. 2009). This supports that further analytical efforts should be made to improve the LODs for the determination of Fe–Cit complexes in plant fluids, taking into account that Fe–Cit complexes could be very sensitive to external conditions and may decompose during chromatography. Also, since the potential occurrence of other Fe compounds along with Fe–Cit complexes could explain the mass balance results found in both standard solutions and xylem sap, further analytical efforts should be made to completely speciate Fe in xylem sap.

The finding that at least an Fe–Cit complex, Fe_3Cit_3, participates in long-distance xylem Fe transport in plants is in line with what is known to occur in other organisms. In humans most of the Fe is usually chelated by transferrin, but the serum of patients with Fe overload disorders may have up to 10 μM Fe not bound to transferrin (at pH 7.4, with 100 μM Cit and an Fe:Cit ratio of 1:10) (Evans et al. 2008). This Fe fraction was proposed to consist of a mixture of oligomeric, dimeric and possibly monomeric Fe species, using a different approach from that used here. The Fe:Cit ratio in serum has also been proposed to control the balance between Fe–Cit species, with Fe:Cit ratios of >1:10 leading to oligomeric and polymeric Fe species and <1:100 leading to monomeric and dimeric Fe species (Evans et al. 2008). This framework is similar to the one we propose here for plant xylem sap, with high Fe:Cit ratios leading to Fe_3Cit_3 and low ratios leading to Fe_2Cit_2. In the bacterial plasma membrane a Fe_2Cit_2 complex is thought to be transported by the coordinated action of FecABCDE proteins (Mahren et al. 2005) where Fe_2Cit_2 binds to FecA in the outer membrane and initiates two independent processes, Fe–Cit transport into the periplasm and transcriptional induction of the *fecABCDE* genes (Yue et al. 2003). Fe–Cit transport by proteins of the CitMHS family has also been recently described in bacteria (Lensbouer et al. 2008).

The idea that Fe could be transported in the plant xylem by organic acids was first suggested many years ago (Rogers 1932), based on the ability of Fe to form stable complexes with organic acids and on the increase in these carboxylates with Fe deficiency (see Abadía et al. 2002 for a review). Pioneering studies by Brown and co-workers proposed that Fe and Cit were associated in some way, in different plant species, from the co-migration of Fe and Cit during electrophoresis and the increase in xylem Cit concentration with Fe deficiency (Brown and Tiffin 1965, Tiffin 1966a, Tiffin 1966b, Tiffin 1970, Clark et al. 1973). The first in silico xylem Fe speciation studies also suggested a major role for Cit in the complexation of Fe in tomato and soybean (White et al. 1981a, White et al. 1981b, Mullins et al. 1986). More recently, in silico studies incorporating the stability constants of other possible Fe chelators (e.g. NA), also support that Cit, rather than NA, could play a major role in Fe xylem transport (von Wirén et al. 1999, López-Millán et al. 2000, López-Millán et al. 2001, Rellán-Álvarez et al. 2008), and this was also supported by ESI-TOFMS direct determination of Fe–NA complexes in standard solutions containing Fe, NA and Cit at typical xylem pH values (Rellán-Álvarez et al. 2008). We have reviewed previous studies reporting xylem Fe and Cit concentrations to explore the likelihood of finding the Fe–Cit complexes in xylem sap (**Supplementary Table 1**). Within a given plant species, Fe:Cit ratios were generally higher in Fe-sufficient than in Fe-deficient plants, and when plants were resupplied with Fe even higher Fe:Cit ratios were found (**Supplementary Table 1**). A comparison of these Fe:Cit ratios with the threshold values proposed in this study with standard solutions (1:10 for >75% Fe_3Cit_3 and 1:75 for >75% Fe_2Cit_2) suggest that the Fe_3Cit_3 complex may occur in a wide range of species regardless of Fe nutrition status. On the other hand, the Fe_2Cit_2 complex may be prevalent in xylem sap samples with Cit concentrations in the mM range, such as those of some Fe-deficient plant species (**Supplementary Table 1**).

Recent molecular evidence also supports that Cit may be involved in long-distance Fe transport. Two *Arabidopsis thaliana* and rice mutants with altered root vasculature Cit transporters show decreases in the xylem sap concentrations of Fe and Cit, increased Fe deficiency symptoms and Fe accumulation in the root (Durrett et al. 2007, Yokosho et al. 2009). Changes found in Fe and Cit concentrations do not support that changes in Fe–Cit speciation may occur, since Fe_3Cit_3 would be expected to occur both in the wild type and mutant genotypes of both species (**Supplementary Table 1**). The phenotype of these mutants could result from a hampered Fe xylem transport, and/or from the decrease in xylem C transport itself, that may in turn impair the ability of the mutant plants to elicit root responses to Fe deficiency. Carbon fixation in roots by phosphoenolpyruvate carboxylase and export in the xylem have been proposed to play a role in the plant responses when leaf C fixation is decreased by Fe deficiency (López-Millán et al. 2000, Zocchi et al. 2007).

The central oxo-bridged tri-Fe(III) core bridged by citrate ligands of the Fe_3Cit_3 complex proposed here to occur in the xylem sap is structurally related to the active sites of numerous polyiron–oxo proteins (Tshuva and Lippard 2004). Di-Fe sites are found in a functionally diverse class of proteins which are

activated by oxygen binding and catalyze hydroxylation, desaturation and epoxidation reactions on a variety of alkyl and aryl substrates. A di-Fe site in ribonucleotide reductase, which participates in DNA biosynthesis, is responsible for the formation of organic radicals, whereas ferritins are important for Fe oxidation, storage and transport (see Tshuva and Lippard 2004 and references therein). This is the first time that a small molecule containing an oxo-bridged tri-Fe center has been reported in a biological system. We may speculate that the oxo-bridge structure may confer redox properties to this plant Fe transport form. In fact, the lowest unoccupied molecular orbital of the complex, obtained by DFT calculations, displays an energy of 1.5 eV, which is rather low for a dianionic species and indicates that the reduction of this trinuclear species could be feasible.

The finding of this negatively double charged, relatively large (750.83 Da) Fe_3Cit_3 complex opens new possibilities to re-examine the long-distance transport of Fe in the plant xylem. Very little is still known about the mechanisms of Fe xylem unloading, although it is accepted that a direct flow of Fe could occur through plasmodesmata into xylem parenchyma cells, and a second mechanism could proceed to the leaf mesophyll apoplast (Kim and Guerinot 2007, Palmer and Guerinot 2009). The molecular size of Fe_3Cit_3, 1.1×0.4 nm, would permit direct passage through plasmodesmata, which usually allows trafficking of molecules <2 nm. Once in the xylem parenchyma cytoplasm, the prevailing pH change would favor ligand exchange reactions with NA, leading to the formation of Fe–NA complexes (von Wirén et al. 1999, Rellán-Álvarez et al. 2008). Xylem Fe unloading can also proceed directly to the apoplast, where the Fe_3Cit_3 complex is also likely to occur [considering apoplast chemical composition (Kim and Guerinot 2007, Palmer and Guerinot 2009)] and will probably function as a suitable substrate for the mesophyll cell leaf plasma membrane Fe(III) chelate reductase. As mentioned above this complex is likely to have redox activity, and the oxo-bridge may play a role in the interaction with the reductase enzyme. In fact, low Fe:Cit ratios—favoring the presence of Fe_3Cit_3—seem to be optimal for leaf plasma membrane Fe(III)-chelate reductase activity, since 20-fold increases were observed when Fe:Cit ratios increased from 1:500 to 1:5 (González-Vallejo et al. 1999). Also, the complex may be a natural substrate of the root Fe(III)-chelate reductase, since it is known that root Cit excretion occurs in many plant species under Fe deficiency (Abadía et al. 2002).

Materials and Methods

Plant culture

Tomato (*Solanum lycopersicum* Mill. cv. 'Tres Cantos') plants were grown in a growth chamber with a photosynthetic photon flux density of 350 µmol m^{-2} s^{-1} photosynthetically active radiation, and a 16/8 h photoperiod, 23/18°C day/night temperature regime. Seeds were germinated and grown in vermiculite for 2 weeks. Seedlings were grown for an additional 2-week period in half-strength Hoagland nutrient solution with 45 µM ^{nat}Fe(III)–ethylenediamine tetraacetic acid (EDTA), and then transplanted to 10-liter plastic buckets (18 plants per bucket) containing half-strength Hoagland nutrient solution, pH 5.5, with either 0 (Fe-deficient plants) or 45 µM Fe(III)–EDTA (Fe-sufficient plants). Throughout this study we use ^{nat}Fe to refer to Fe with the natural isotopic composition: 5.85, 91.75, 2.12 and 0.28% of ^{54}Fe, ^{56}Fe, ^{57}Fe and ^{58}Fe, respectively. After 10 d, Fe-deficient plants were resupplied with Fe by transfer to nutrient solution with Fe [45 µM either ^{nat}Fe(III)–*o,o*EDDHA, ^{54}Fe(III)–*o,o*EDDHA or ^{nat}Fe(III)–EDTA]. Iron-54 was purchased as Fe_2O_3 (98% Fe, 95% ^{54}Fe; Cambridge Isotope Labs, Andover, MA, USA). Xylem was sampled from Fe-deficient, Fe-sufficient and Fe-deficient plants resupplied with Fe for 6, 12 and 24 h.

Xylem sap sampling

Tomato xylem sap was sampled using the detopping technique (López-Millán et al. 2009). Plant shoots were cut just below the first true leaf using a razor blade, and xylem sap was left to exude. The sap of the first 5 min was discarded to avoid contamination, the surface was washed with distilled water and blotted dry, and sap was then directly collected for 20 min using a micro-pipet and maintained in Eppendorf tubes kept on ice. Immediately after sample collection, the pH of the samples was measured with a Biotrode pH microelectrode with Idrolyte electrolyte (Metrohm, Herisau, Switzerland), tested for cytosolic contamination as indicated below and kept frozen at −80°C until further analysis. All samples were assessed for cytosolic contamination using c-mdh (EC 1.1.1.37) as a cytosolic contamination marker (López-Millán et al. 2000), and no contamination was found. Before analysis, samples were thawed and diluted 2-fold with 10 mM ammonium acetate in methanol at pH 6.8. The actual pH value of this organic mixture is estimated to be 5.5 (Canals et al. 2001). Then, samples were vortexed, centrifuged at 12 000 × *g* for 2 min and the supernatant immediately analyzed.

Fe–Cit standard solutions preparation

Iron-Cit standard solutions at different Fe:Cit ratios were prepared by adding the appropriate amounts of Fe (^{nat}Fe or ^{54}Fe) to Cit solutions prepared in 10 mM ammonium acetate at pH 5.5. Fe–Cit solutions were gently vortexed, diluted 2-fold with 10 mM ammonium acetate in methanol at pH 6.8 and immediately analyzed. The pH value of this organic mixture is estimated to be 5.5 (Canals et al. 2001).

Analysis of Fe complexes by HPLC coupled to electrospray ionization MS

Chromatographic separation was performed on a modified Alliance 2795 HPLC system (Waters, Mildford, MA, USA). The fluidics system, with the exception of the pump head, was built with PEEK, and a Ti needle was used to minimize metal contamination. Different HPLC conditions were tested, including

several column types, elution programs and organic solvents (acetonitrile and methanol). Final HPLC analysis conditions were as follows. The autosampler was kept at 4°C and the column compartment temperature was set at 30°C. Injection volume was 20 µl, and a ZIC-pHILIC, 150×2.1 mm, 5 µm column (Sequant, Umea, Sweden) was used with a flow rate of 100 µl min^{-1}. The mobile phase was built using two eluents: A (10 mM ammonium acetate in water at pH 5.5) and B (10 mM ammonium acetate in methanol at pH 6.8). All mobile phase chemicals were LC-MS grade (Riedel-de Haën, Seelze, Germany). For separation, an initial equilibration time of 80% B and 20% A (from min 0 to 5) was followed by a linear gradient from 80 to 20% B (from min 5 to 20). This mobile phase composition was held for 15 min, then changed linearly to the initial conditions for 10 min, and kept as such for another 15 min. Total run time per sample was 1 h. The pH values during the HPLC run ranged from 5.3 to 6.2 depending on organic mixture composition of the mobile phase (Canals et al. 2001). Injections of 5 mM Cit (20 µl) were carried out between samples to minimize Fe cross-contamination.

High-resolution MS analysis was carried out with a micrOTOF II ESI-TOFMS apparatus (Bruker Daltoniks GmbH, Bremen, Germany) in the 50–1000 m/z range. The micrOTOF II was operated in negative mode at 3000 and −500 V capillary and end-plate voltages, respectively. After optimization, capillary exit, skimmer 1 and hexapole RF voltages were set at −57.1, −39.1 and 145.2 V, respectively. Nebulizer gas (N_2) pressure was kept at 2.0 bar and drying gas (N_2) flow was set at 8.0 liter min^{-1} with a temperature of 180°C. Mass calibration was carried out with 10 mM Li-formate solution using a syringe pump (Cole-Parmer Instruments, Vernon Hills, IL, USA). In each HPLC run mass calibration was carried out by on-line injection of 20 µl of Li-formate at 2 min. Molecular formulae were assigned based on (i) exact molecular mass, with errors <10 ppm, and (ii) the SigmaFit algorithm, with a threshold tolerance value of 0.03 SigmaFit values (Ojanperä et al. 2006). Appropriate isotopic abundances were used for the calculations. The system was controlled with the software packages MicrOTOF Control v.2.2 and HyStar v.3.2 (Bruker Daltonics). Data were processed with Data Analysis v.3.4 software (Bruker Daltonics).

Analysis of Fe and Fe complexes by ICP-MS

ICP-MS analysis was carried out with a Q-ICP-MS instrument (7500ce; Agilent Technologies, Tokyo, Japan). The instrument was fitted with an octapole collision cell system located between the ion lenses and a quadrupole MS analyzer for removal of polyatomic interference. In this study He was used as a collision gas. In addition, O_2 (7%) was added to the plasma by an additional mass flow controller to remove C excess, and to prevent it from condensing on the interface and ion lenses due to the high organic content of the mobile phase during HPLC separation (Woods and Fryer 2007). Platinum interface cones were used to allow the addition of O_2. The Q-ICP-MS instrument was operated with a RF power of 1500 W and cooling, sample and make-up gas flows of 15, 0.9 and 0.2 liter min^{-1}, respectively. The collision cell was operated with a He gas flow of 4.2 ml min^{-1} and octapole bias and QP bias voltages of −18.0 and −16.0 V, respectively. The torch position and ion lens voltage settings were optimized daily for maximum sensitivity with a 1 ng g^{-1} Li, Co, Y, Tl and Ce mixture in 1% (w/w) HNO_3 solution. A solution of 1% (w/w) HNO_3 was also used to check the background level caused by polyatomic Ar interference. The possible contribution of isobaric interference of ^{54}Cr and ^{58}Ni in the determination of the ^{54}Fe and ^{58}Fe isotopes was corrected mathematically by measuring the ion signals at masses 52 for Cr and 60 for Ni and assuming natural abundances as reported by IUPAC (De Laeter et al. 2003). Mass bias correction was carried out by measuring the isotope ratios of the ^{nat}Fe standard and calculating the mass bias factor (K) with an exponential model (Rodríguez-Castrillón et al. 2008). The ICP-MS instrument was controlled with the software packages ICP-MS ChemStation v.B.03.04 and ICP-MS Chromatographic v.B.03.04 (Agilent Technologies).

For the ICP-MS analysis of ^{54}Fe–citrate complexes in standard solutions and xylem sap samples, a chromatographic separation was performed on an Agilent 1100 chromatographic system (Agilent Technologies) using the method described for HPLC–ESI-TOFMS analysis. Iron-54 and ^{nat}Fe quantification was carried out by post-column isotope dilution analysis (IDA) (Rodríguez-González et al. 2005) with a 20 ng g^{-1} ^{57}Fe in EDTA solution continuously introduced at 0.10 g min^{-1} through a T piece connected to the end of the column and before the plasma entrance. Iron-57 was purchased as Fe_2O_3 (98% Fe, 95% ^{57}Fe; Cambridge Isotope Labs). The ICP-MS intensity chromatograms (counts s^{-1}) were converted into Fe molar flow chromatograms (^{54}Fe nmol min^{-1}) using the isotope pattern deconvolution (IPD) equations described elsewhere (Rodríguez-Castrillón et al. 2008, González Iglesias et al. 2009). Accurate isotope abundances of the ^{57}Fe and ^{54}Fe enriched solutions were determined by direct ICP-MS injections and used for these calculations. Isotope abundances (% ^{54}Fe, ^{56}Fe, ^{57}Fe and ^{58}Fe) were 0.14, 4.74, 94.63 and 0.49 in ^{57}Fe-enriched solutions and 99.67, 0.25, 0.01 and 0.06 in ^{54}Fe-enriched solutions.

Total ^{54}Fe and ^{nat}Fe determinations in xylem sap samples were carried out by direct ICP-MS injections and IDA, spiking known amounts of the characterized ^{57}Fe-enriched solution into the samples acidulated with HNO_3. Also, the mathematical IPD procedure described elsewhere (Rodríguez-Castrillón et al. 2008, González Iglesias et al. 2009) was used.

Other determinations

Citrate was analyzed by HPLC (Waters Alliance 2795) using a Supelcogel H 250×4.6 mm column. Analyses were performed isocratically at a flow rate of 200 µl min^{-1} and at a temperature of 30°C. The mobile phase was 0.1 % formic acid. Detection was performed by ESI-TOFMS (micrOTOF II; Bruker Daltonics) at 191.0 m/z. Quantification was carried out by external calibration with internal standarization (with [^{13}C]4-L-malic acid; Cambridge Isotope Laboratories). The Fe(III) chelate of *o,o*EDDHA was determined as described elsewhere (Orera et al. 2009).

Iron–citrate complex molecular modeling

All theoretical calculations were performed by using the Gaussian 03 program (Frisch et al. 2003). The molecular geometry of $(Fe_3OCit_3)^{2-}$ was optimized assuming C_{3h} symmetry. The chemistry model used consisted in the Becke's three-parameter exchange functional combined with the LYP correlation functional (B3LYP) (Becke 1993) and the LanL2DZ basis set as indicated in the Gaussian 03 program (Frisch et al. 2003). In order to achieve the convergence of the wavefunction, an initial guess was obtained using the same chemistry model on the closed shell $(Fe_3OCit_3)^{2-}$ species.

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Supplementary Table I. Xylem sap pH, Fe and citrate concentrations, and Fe:Cit ratios found in previous studies under different Fe status. The most likely Fe-Cit forms are indicated with ● symbols.

	Fe status[a]	Fe in solution (μM)[b]	pH	Fe (μM)	Cit (μM)	Fe:Cit	Fe_3Cit_3[c]	Fe_2Cit_2[c]
Sunflower (Tiffin, 1966)	+Fe	10 + 10	-	2	30	1:15	●	●
	-Fe/+Fe	0.01 + 10	-	31	890	1:29	●	●
Sunflower (Tiffin, 1966)	-Fe	1 + 1	-	8	200	1:25	●	●
	-Fe/+Fe	1 + 50	-	465	460	1:1.0	●	
Tomato (Tiffin, 1966)	-Fe	1 + 2	-	28	72	1:2.6	●	
	-Fe/+Fe	1 + 10	-	131	135	1:1.0	●	
Soybean (Tiffin, 1966)	-Fe	1 + 0.5	-	10	400	1:40	●	●
	-Fe/+Fe	1+ 10	-	290	710	1:2.5	●	
Soybean (Tiffin, 1970)	-Fe	1 + 2.5	-	34	120	1:3.6	●	
Zea mays L WF9 (Clark et al., 1973)	-Fe	18 + 0	-	57	120	1:2	●	
Zea mays L ys1/ys2 (Clark et al., 1973)	-Fe		-	9	430	1:48	●	●
Soybean (White et al., 1981)	+Fe	10	6.1	6	1700	1:283		●
Tomato (White et al., 1981)	+Fe		6.4	7	300	1:43	●	●
Faba bean (Nikolic and Römheld, 1999)	+Fe	100	5.5	12	100	1:8	●	
	-Fe	2	5.5	4	2600	1:650		●
	-Fe	1 + HCO_3^-	5.5	3	2300	1:767		●
Sugar beet (López-Millán et al., 2000)	+Fe	45	6.0	6	200	1:36	●	●
	-Fe	0 + HCO_3^-	5.7	2	4700	1:2474		●
Pear trees (Larbi et al., 2003)	-Fe	field-grown	6.8	3	780	1:260		●
	-Fe/+Fe		6.5	4	470	1:118		●
Peach trees (Larbi et al., 2003)	-Fe		6.6	2	250	1:125		●
	-Fe/+Fe		6.4	5	40	1:8	●	
A. thaliana WT (Durrett et al., 2007)	+Fe	100	-	11	90	1:8	●	
A. thaliana frd3-1 (Durrett et al., 2007)	+Fe		-	5	55	1:11	●	●
O. sativa WT (Yokosho et al., 2009)	-Fe	2	-	6	185	1:31	●	●
	+Fe	10	-	13	135	1:10	●	●
O. sativa FRDL1 KO (Yokosho et al., 2009)	-Fe	2	-	5	80	1:16	●	●
	+Fe	10	-	9	55	1:65	●	●

[a]Iron-sufficient, Fe-deficient and Fe-deficient, Fe-resupplied plants are labeled as +Fe, -Fe and -Fe/+Fe.
[b]When plants were precultured in a different nutrient solution the Fe concentration is indicated as A + B, where A is the Fe concentration in the preculture solution and B is the Fe concentration in the final one. Citrate, Fe and pH xylem sap values correspond to plants grown in the final nutrient solution.
[c]Iron to citrate concentration ratios higher than 1:10 will lead to Fe_3Cit_3, ratios lower than 1:75 will lead to Fe_2Cit_2, and ratios between 1:10 and 1:75 will lead to both complexes.

Supplementary Table II. Atom coordinates of the Fe_3Cit_3 complex. The center numbers a
indicated in Supplementary Fig. 6. All values are expressed in Amstrongs.

Center number	Atomic number	Coordinates		
		X	Y	Z
1	8	0.000000	0.000000	0.000000
2	26	1.910259	0.007702	0.000000
3	26	-0.948460	-1.658184	0.000000
4	26	-0.961800	1.650482	0.000000
5	8	-2.478861	1.197270	1.251661
6	8	-1.916080	-0.994839	1.526843
7	6	-2.789751	-0.038554	1.417397
8	8	-2.478861	1.197270	-1.251661
9	8	-1.916080	-0.994839	-1.526843
10	6	-2.789751	-0.038554	-1.417397
11	8	0.096485	2.156794	1.526843
12	8	2.276297	1.548122	1.251661
13	6	1.361487	2.435272	1.417397
14	8	2.276297	1.548122	-1.251661
15	8	0.096485	2.156794	-1.526843
16	6	1.361487	2.435272	-1.417397
17	8	0.202565	-2.745392	1.251661
18	8	1.819596	-1.161955	1.526843
19	6	1.428264	-2.396718	1.417397
20	8	0.202565	-2.745392	-1.251661
21	8	1.819596	-1.161955	-1.526843
22	6	1.428264	-2.396718	-1.417397
23	6	2.561794	-3.405276	1.309731
24	1	3.240187	-3.292325	2.161717
25	1	2.181368	-4.430083	1.274604
26	6	2.561794	-3.405276	-1.309731
27	1	3.240187	-3.292325	-2.161717
28	1	2.181368	-4.430083	-1.274604
29	6	3.411223	-3.161585	0.000000
30	6	1.668158	3.921217	1.309731
31	1	1.231144	4.452247	2.161717
32	1	2.745881	4.104162	1.274604
33	6	1.668158	3.921217	-1.309731
34	1	1.231144	4.452247	-2.161717
35	1	2.745881	4.104162	-1.274604
36	6	1.032402	4.534998	0.000000
37	6	-4.229952	-0.515941	1.309731
38	1	-4.927249	0.325922	1.274604

39	1	-4.471331	-1.159922	2.161717
40	6	-4.443624	-1.373413	0.000000
41	6	-4.229952	-0.515941	-1.309731
42	1	-4.927249	0.325922	-1.274604
43	1	-4.471331	-1.159922	-2.161717
44	6	4.288980	-1.772224	0.000000
45	8	3.822713	-0.553188	0.000000
46	8	5.545710	-2.022058	0.000000
47	8	4.404176	-4.226855	0.000000
48	1	5.277408	-3.731650	0.000000
49	6	-3.679281	-2.828253	0.000000
50	8	-2.390431	-3.033973	0.000000
51	8	-4.524009	-3.791697	0.000000
52	8	-5.862652	-1.700701	0.000000
53	1	-5.870407	-2.704545	0.000000
54	6	-0.609699	4.600477	0.000000
55	8	-1.432282	3.587160	0.000000
56	8	-1.021702	5.813755	0.000000
57	8	1.458476	5.927556	0.000000
58	1	0.592999	6.436194	0.000000

Supplementary Table III. Atom coordinates of the Fe_2Cit_2 complex. The center numbers are indicated in Supplementary Fig. 8. All values are expressed in Amstrongs.

Center number	Atomic number	Coordinates		
		X	Y	Z
1	26	-0.649031	-1.269867	0.345147
2	8	-2.273378	-2.137284	-0.205427
3	8	0.365552	-2.585555	-0.668604
4	8	0.95098	-0.285869	0.693017
5	8	-0.95193	0.285427	-0.695264
6	8	-1.678777	-0.343245	1.678666
7	8	1.791792	-4.35806	-0.40878
8	8	-4.352662	-2.257098	-1.131281
9	8	-3.451755	1.12418	1.784734
10	6	-3.328889	-1.594025	-0.806204
11	6	-3.296482	-0.082957	-1.15242
12	6	-2.288066	0.78429	-0.363277
13	6	-2.387041	2.278805	-0.694062
14	6	1.471176	-3.157399	-0.190886
15	6	-2.522466	0.551273	1.173632
16	26	0.648976	1.270087	-0.344755
17	6	-1.471616	3.157646	0.187817
18	6	2.385836	-2.279492	0.692795
19	6	2.287712	-0.784723	0.363143
20	8	2.27388	2.137016	0.206819
21	8	-0.366502	2.585828	0.666859
22	8	1.680033	0.344148	-1.678471
23	8	-1.791055	4.359045	0.403416
24	6	3.295229	0.081948	1.153975
25	6	2.523537	-0.550281	-1.173588
26	6	3.328066	1.593477	0.809266
27	8	3.453849	-1.122397	-1.783827

28	8	4.351311	2.256054	1.137158
29	1	-3.057366	-0.00083	-2.221894
30	1	-4.312482	0.29819	-1.002936
31	1	-2.105865	2.434717	-1.744268
32	1	-3.413489	2.627254	-0.54769
33	1	3.412414	-2.627896	0.54719
34	1	2.103544	-2.436238	1.742562
35	1	4.311288	-0.299075	1.004555
36	1	3.055736	-0.001408	2.223267
37	8	0.667394	2.723017	-1.802866
38	8	-0.665955	-2.723827	1.802424
39	1	1.096799	3.434919	-1.276741
40	1	1.331386	2.256401	-2.364666
41	1	-1.330323	-2.257944	2.364365
42	1	-1.094422	-3.436212	1.276321

Supplementary Figure Legends.

Supplementary Figure 1. ESI-TOFMS mass spectra of a ^{nat}Fe-Cit standard solution (Fe:Cit ratio 1:10, 100 µM ^{nat}Fe, pH 5.5, in 50% mobile phase B) before (A) and after electrospray ionization optimization (B). Isotopic signatures of the $[CitH]^-$, $[Fe_3Cit_3H]^{2-}$ and $[Fe_3OCit_3H_3]^{2-}$ molecular ions are shown in the panel insets.

Supplementary Figure 2. HPLC-UV chromatograms obtained with Agilent 1100 (A) and Waters 2795 (B) HPLC systems of an Fe-Cit standard solution (Fe:Cit ratio 1:10, 100 µM Fe, pH 5.5 diluted in 50% mobile phase B) showing peaks corresponding to Fe_2Cit_2 and Fe_3Cit_3 complexes.

Supplementary Figure 3. HPLC-ESI-TOFMS chromatograms of 5 mM citrate (A), 100 µM Fe (III)-NA (B) and 100 µM Fe(III)-DMA (C) standard solutions. Chromatograms show the $[CitH]^-$ (A), $[Fe(III)\text{-}NA+Cl]^-$ (B) and $[Fe(III)\text{-}DMA]^-$ (C) ions at m/z values 191.0, 391.0 and 356.0 (±0.1), respectively.

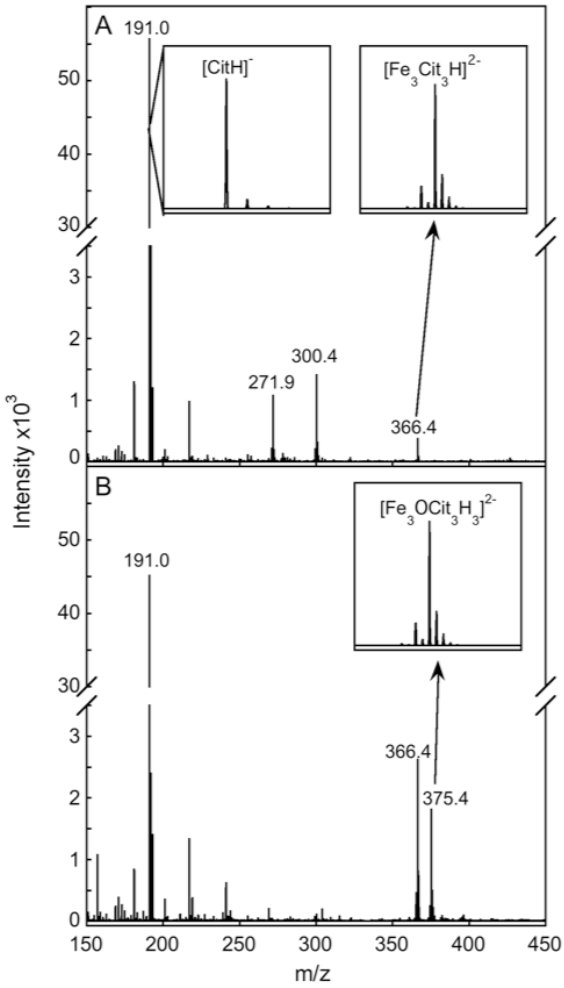
Supplementary Figure 4. Experimental (A, C, E and G) and theoretical (B, D, F and H) isotopic signatures of the molecular ions associated to Fe_2Cit_2 and Fe_3Cit_3, $[Fe_2Cit_2H]^-$ and $[Fe_3Cit_3H]^{2-}$, respectively. Experimental data are zoomed ESI-TOF mass spectra of the Fe_2Cit_2 and Fe_3Cit_3 chromatographic peaks found when using ^{54}Fe (A, E) and ^{nat}Fe (C, G).

Supplementary Figure 5. Effect of the Fe:Cit ratio on the Fe_2Cit_2 and Fe_3Cit_3 balance, HPLC-ICP-MS (A-D) and HPLC-ESI-TOFMS (E-H) chromatograms of ^{54}Fe-Cit standard solutions with Fe:Cit ratios 1:1, 1:10, 1:100 and 1:500 (100 µM ^{54}Fe, pH 5.5 in 50% mobile phase B) showing peaks corresponding to Fe_2Cit_2 and Fe_3Cit_3 complexes. HPLC-ESI-TOFMS traces were extracted at m/z values 241.93 and 484.87 (±0.05), corresponding to $[Fe_2Cit_2]^{2-}$ and $[Fe_2Cit_2H]^-$, respectively, (for Fe_2Cit_2, solid line) and 363.40 and 372.40 (±0.05), corresponding to $[Fe_3Cit_3H]^{2-}$ and $[Fe_3OCit_3H_3]^{2-}$, respectively, (for Fe_3Cit_3, dotted line).

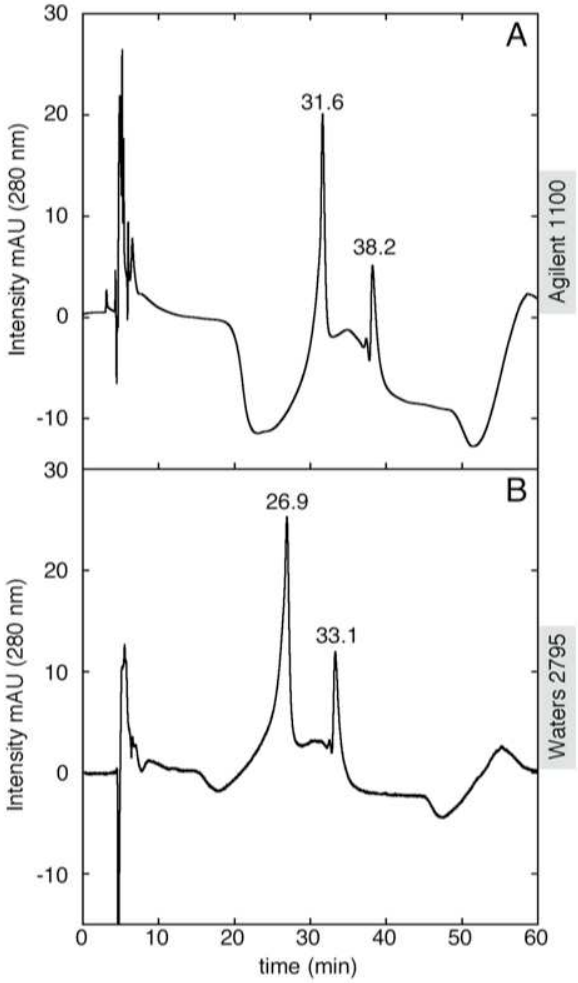
Supplementary Figure 6. Proposed structure for the Fe_3Cit_3 indicating the center numbers (See atom coordinates in Supplementary Table I). Iron, oxygen, carbon and hydrogen atoms are shown in purple, red, green and white, respectively.

Supplementary Figure 7. Proposed structure for the Fe_2Cit_2 complex. Iron, oxygen, carbon and hydrogen atoms are shown in purple, red, green and white, respectively.

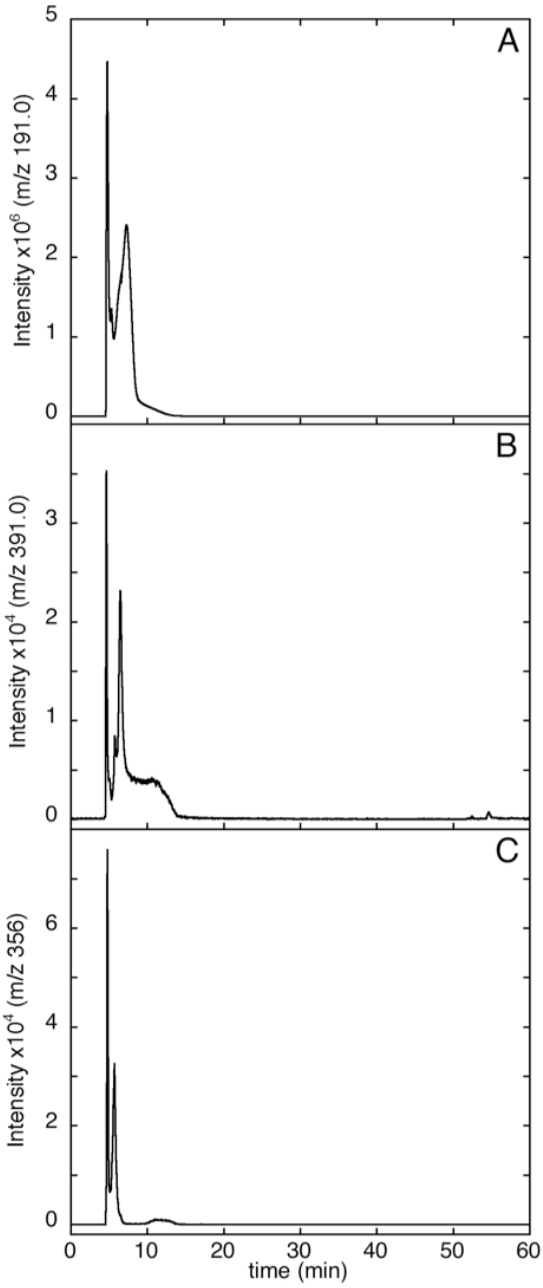
Supplementary Figure 1



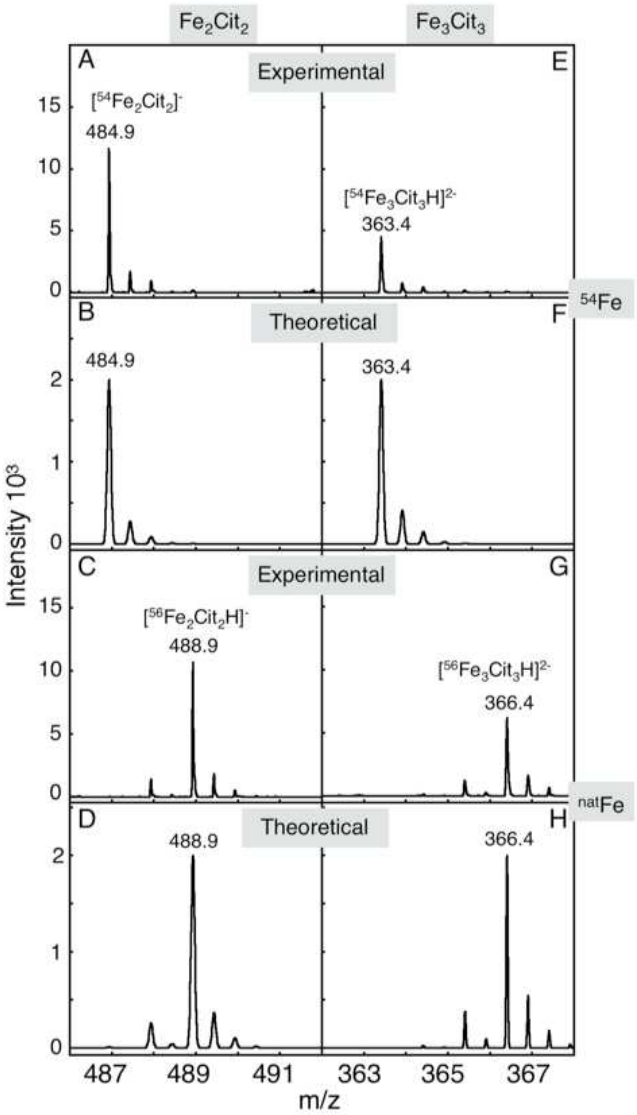
Supplementary Figure 2



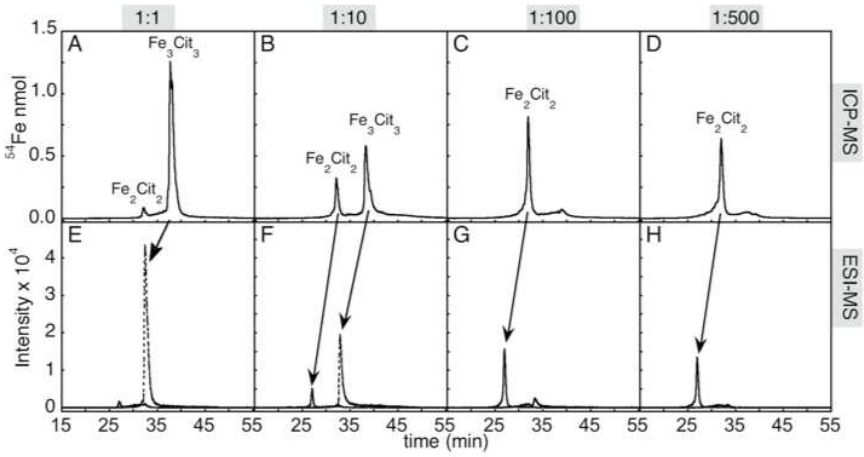
Supplementary Figure 3



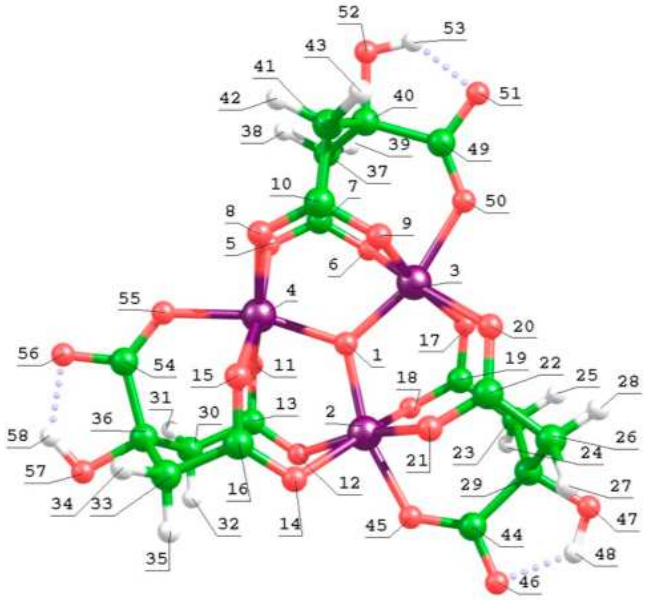
Supplementary Figure 4



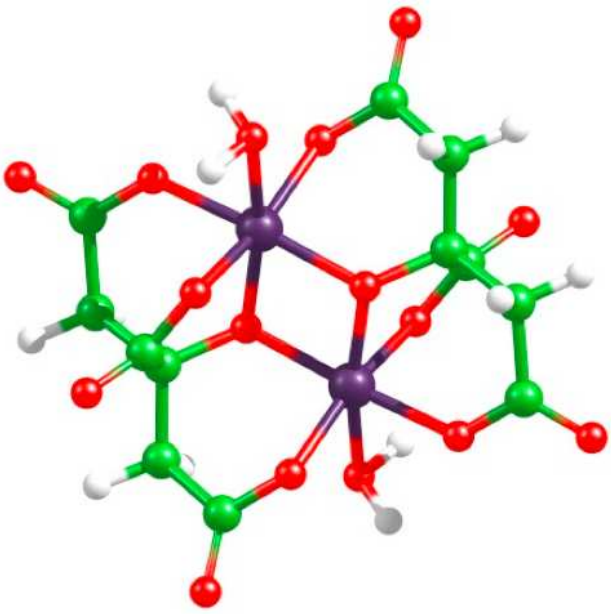
Supplementary Figure 5



Supplementary Figure 6



Supplementary Figure 7



Supplementary Figure 8. Proposed structure for the Fe_2Cit_2 indicating the center numbers (See atom coordinates in Supplementary Table III). Iron, oxygen, carbon and hydrogen atoms are shown in purple, red, green and white, respectively.

Supplementary Figure 8