UNIVERSIDAD AUTÓNOMA DE MADRID

PROGRAMA DE DOCTORADO EN QUÍMICA AGRÍCOLA

TESIS DOCTORAL

COMPLEJOS DE MICRONUTRIENTES: CARACTERIZACIÓN Y FACTORES QUE INFLUYEN EN SU EFICACIA COMO FERTILIZANTES

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MADRID, 2019

UNIVERSIDAD AUTÓNOMA DE MADRID

FACULTAD DE CIENCIAS

DEPARTAMENTO DE QUÍMICA AGRÍCOLA Y BROMATOLOGÍA

COMPLEJOS DE MICRONUTRIENTES: CARACTERIZACIÓN Y FACTORES QUE INFLUYEN EN SU EFICACIA COMO FERTILIZANTES

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Madrid, 2019

“La importancia en la ciencia no es tanto obtener nuevos datos,

sino descubrir nuevas formas de pensar sobre ellos”.

William Lawrence Bragg (1890-1971)

Agradecimientos

En primer lugar quiero agradecer al Dr. Juan José Lucena Marotta por haberme dado la oportunidad de pertenecer a su grupo de trabajo, por sus conocimientos brindados, por su apoyo constante y su paciencia durante mi formación como investigador y en el desarrollo de la Tesis. Así como también por la confianza que me ha tenido, pese a los altibajos científicos.

A la Dra. Sandra López Rayo por su ayuda y conocimientos compartidos, así como por sus consejos y participación coactiva en la realización de ésta Tesis Doctoral. Además de sus aportaciones científicas, las cuales han contribuido al mejoramiento de este trabajo

Al resto de profesores y colegas del Departamento de Química Agrícola y Bromatología: Elvira, Lourdes, Enrique, Felipe, Agustín, Jesús, Eduardo, Sandra, Silvia, Vanessa, Esperanza y Virginia, que siempre han tenido una sonrisa y/o palabra amable que han hecho más amenos todos estos años.

A todos esos compañeros que encontré en el despacho 350, los cuales con el paso del tiempo han llegado a convertirse en mucho más que eso. Gracias por ayudarme dentro y fuera del trabajo, por esos buenos ratos que hemos pasado y esos pequeños convivios. Todo esto no hubiera sido posible sin la amistad de todos ustedes: Teresa, Clara, Alex, Raquel, Rafa, Begoña, Alejandra, Sara, Fernando, Javad, Meriem.

Al Dr. Carlos Alexander Lucho Constantino por el apoyo y conocimientos brindados desde el inicio de mi carrera investigadora, por apoyarme en los momentos difíciles y por alentarme a seguir adelante. Al resto de miembros del Centro de Investigaciones Químicas de la Universidad Autónoma del Estado de Hidalgo, en especial a la Dra. Rosa Icela Beltrán, la Dra. Claudia Coronel, la Dra. Gabriela Vázquez y la Dra. Georgina Martínez que también han contribuido en mi formación y me permitieron formar parte de su grandioso equipo de trabajo.

Al Dr. Juan M. Herrera por sus conocimientos y ayuda brindados, los cuales contribuyeron en mi motivación por nuevos campos de investigación.

A mis amigos Areli, Karina, Nahum, Miguel, Lili, Mariana, Laura, Miriam y Lucía que también han contribuido indirectamente en este nuevo logró, a través de su amistad y apoyo durante todos estos años.

A Francisco Callejas porque ha llenado cada momento de alegría y aprendizajes, por hacer más llevadero este tiempo con su apoyo y consejos. Por motivarme en esos

momentos difíciles y por hacerme ver que todo esfuerzo tiene su recompensa. Por devolverme la confianza en mí, todas esas veces que la vi perdida. Porque indirectamente y directamente también ha contribuido en este gran logró, el cual solo inició como un pequeño sueño y comenzó a hacerse realidad en el momento en que llegamos a este lugar.

A mi familia que son el motor que me impulsa a dar lo mejor de mí en cada proyecto de mi vida y por estar conmigo en todo momento de mi vida compartiendo mis triunfos y fracasos. También, por su cariño incomparable y por apoyarme en cada proyecto de mi vida. Porque sé, que aunque esta meta fue un reto difícil para todos, cuando tome la decisión confiaron en mí y me apoyaron hasta el último momento. Y en especial a mi padre y a mi abuela, que con su cariño también influyeron en este logro.

A todos gracias por su apoyo

La presente Tesis Doctoral se ha realizado a través de la financiación de la siguiente beca:

Consejo Nacional de Ciencia y Tecnología (CONACYT). Beca predoctoral No. 278934 del Programa de Becas CONACYT al extranjero 2015 otorgada a través del Consejo de Ciencia, Tecnología e Innovación de Hidalgo (CITNOVA).

Y gracias a la financiación de los siguientes proyectos:

Proyecto FUAM 500063: Determinación de la capacidad complejante de los lignosulfonatos (LS). Financiado por Tembec Innovation, Francia.

Proyecto AGL2013-44474-R: ”Correctores biodegradables de deficiencias de Fe, Mn y Zn. Nuevos diseños y técnicas de evaluación” Financiado por el Ministerio de Economía y Competitividad.

Proyecto RTI2018-096268-B-I00: “Nuevos fertilizantes de micronutrientes y bioestimulantes: criterios de eficacia y mecanismos de acción”. Ministerio de Ciencia, Innovación y Universidades.

Proyecto AGRISOST-CM S2018/BAA-4330. “Tecnología destinada a la sostenibilidad de los sistemas agrícolas”. Financiado por la Comunidad Autónoma de Madrid y por el Fondo Social Europeo (FSE) y por el Fondo Europeo de Desarrollo Regional (FEDER) para el período de programación 2014-2020.

RESUMEN

Las deficiencias de micronutrientes en suelo calizo son un problema nutricional común en cultivos sensibles. La aplicación de quelatos sintéticos es la práctica más eficiente para afrontar este problema, pero el riesgo ambiental causado por su efecto recalcitrante y su capacidad para movilizar metales pesados han promovido la búsqueda de alternativas biodegradables y de bajo costo. El uso de lignosulfonato (LS), heptagluconato (G7) o gluconato (G6) tiene un bajo impacto ambiental, pero en la mayoría de los casos son menos efectivos que los quelatos debido a su baja estabilidad. Es por ello que se necesita profundizar en los factores relacionados con la eficacia de estos complejos.

En esta Tesis doctoral, se evaluaron los parámetros fisicoquímicos que influyen en la eficacia de los complejos de LS, G7 y G6 para proveer los micronutrientes Fe, Mn y Zn a los cultivos mediante técnicas de caracterización química y ensayos biológicos. Los complejos de Fe, Mn y Zn fueron analizados tanto por técnicas espectroscópicas como por cromatografía de filtración en gel (GFC) y cromatografía de exclusión por tamaño de alta resolución (HPSEC). La capacidad de los complejos G7 y G6 aplicados en diferentes relaciones de metal:ligando para proveer Fe o Mn a plantas deficientes fue evaluado y comparado con agentes quelantes sintéticos como HBED y EDTA, bajo condiciones de hidroponía y suelo calizo.

La caracterización fisicoquímica demostró que la eficacia de los complejos depende de varios factores, tales como: **1)** el tipo de metal complejado, **2)** el tipo de ligando, **3)** los grupos funcionales presentes en la estructura del ligando, **4)** la distribución del peso molecular en la formación del complejo y **5)** la ionización de los grupos funcionales y los sitios de unión, los cuales a su vez dependen del pH. El fraccionamiento de los complejos de Fe(III) por GFC y HPSEC indicó la formación de compuestos de alto peso molecular como resultado de la presencia de estructuras polinucleares de Fe(III), mientras que para los complejos de Mn(II) y Zn(II) no se observó formación de agregados. Los ensayos biológicos demostraron que el G7 presenta una mayor eficacia como fertilizante que el G6 para proveer Fe y Mn a planta, tanto en hidroponía como en suelo, y una alternativa biodegradable a los quelatos sintéticos formados con EDTA y HBED. Además, la capacidad fertilizante de los complejos de G7 depende de la relación metal:ligando y de la dosis aplicada.

Así por tanto, esta tesis contribuye a mejorar el conocimiento en la selección de los ligandos y los factores que influyen en su aplicación como fertilizantes, especialmente bajo condiciones de suelo calizo.

ABSTRACT

Micronutrient deficiencies are a common nutritional problem of sensitive crops grown in calcareous soil. The application of synthetic chelates is the most efficient practice to overcome this problem, but the environmental risk caused by their recalcitrant effect and capacity to mobilize heavy metals has promoted the search of biodegradable, low-cost and environmental-friendly alternatives. The use of lignosulfonate (LS), heptagluconate (G7) or gluconate (G6) has a low environmental impact, but in most cases, they are less effective than chelates due to its low stability. Therefore, it is necessary to investigate the factors involved in its efficacy.

In this Doctoral Thesis, the physicochemical parameters that influence the efficacy of the complexes of LS, G7 and G6 to provide Fe, Mn and Zn to crops were evaluated by means chemical techniques of characterization and biological assays. Complexes of Fe, Mn, and Zn were analyzed by spectroscopic techniques, along with gel filtration chromatography (GFC) and high-performance size exclusion chromatography (HPSEC). The ability of G7 and G6 complexes at different metal:ligand ratios to provide Fe or Mn to deficient plants was evaluated and compared to the chelating agents HBED and EDTA under hydroponics and calcareous soil conditions.

The physicochemical characterization pointed out that the efficacy of the complexes depends on several factors such as **1)** the type of complexed metal, **2)** the ligand type, **3)** the functional groups present in the structure of the ligand, **4)** the molecular weight distribution in the complex formed, and **5)** the pH-dependency of the ionization of the functional groups and the binding sites. The fractionation by GFC and HPSEC indicated the formation of high molecular weight compounds in the Fe (III) complexes as a result of the Fe (III) polynuclear structures formed, whereas the Zn (II) and Mn (II) complexes did not form aggregates. The biological assays demonstrated that the G7 presented a higher efficacy as fertilizer than G6 to provide Fe and Mn in hydroponics and calcareous soil, and a biodegradable alternative to synthetic chelates as those formed with EDTA and HBED. Besides, the fertilizer capacity of the G7 complexes depended on the metal:ligand ratio and the dose applied.

Therefore, this thesis contributes to improving the knowledge in the selection of the ligands and the factors that influence its application as fertilizers, especially under calcareous soil conditions.

ABREVIATURAS

AAS	Espectroscopía de absorción atómica, atomic absortion spectroscopy
av M_n	Peso molecular promedio en número, the number average molecular weight.
av MW	Peso molecular promedio en peso, the weight average molecular weight
DAT	Días después del tratamiento, days after treatment
DW	Peso seco, dry weight
E1	Lignosulfonato de eucalipto, eucalyptus lignosulfonate
EDTA	Ácido etilendiaminotetraacético, ethylenediaminetetraacetic acid
FTIR	Espectroscopía de infrarrojos por transformada de Fourier, Fourier transform infrared spectroscopy
G6	Gluconato, gluconate
G7	Heptagluconato, heptagluconate
GFC	Cromatografía de filtración en gel, gel filtration chromatography
HBED	Ácido N,N'-bis (2-hidroxibenzil) etilendiamino-N,N'-diacético, N,N′-di(2-hydroxybenzyl)ethylenediamine-N,N′-diacetic acid
HMW	Alto peso molecular, high molecular weight
HPSEC	Cromatografía de exclusión por tamaño de alta resolución, high-performance size exclusion chromatography.
LMW	Bajo peso molecular, low molecualr weight
LS	Lignosulfonato, lignosulfonate
LSA	Ácido lignosulfónico, lignosulfonic acid
MCC	Máxima capacidad de complejación, maximum complexing capacity
MWD	Distribución del peso molecular, molecular weight ditribution
NMR	Resonancia magnética nuclear, nuclear magnetic resonance
P1, P2, P3	Lignosulfonatos de amonio, ammonium lignosulfonate
P4	Lignosulfonato de magnesio, magnesium lignosulfonate
P5	Lignosulfonato de potasio, potassium lignosulfonate
PA	Parte aérea de la planta, shoot
S1	Lignosulfonato de abeto, spruce lignosulfonate
SEC	Cromatografía de exclusión por tamaño, size exclusión chromatography
SN	Disolución nutritiva, nutrient solution
T_o	Tiempo de exclusión, exclusion time
T_p	Tiempo total de permeación, total permeation time

TR	Translocación, translocation
V_o	Volumen de exclusión, exclusion volume
V_p	Volumen total, total permeation volumen

ÍNDICE

I. INTRODUCCIÓN

1.1 Importancia de los micronutrientes metálicos en los cultivos

Los ochos micronutrientes esenciales en el desarrollo de las plantas, se caracterizan por que son requeridos en bajas concentraciones (5-100 mg Kg^{-1}) (Alloway, 2008; Kumar *y col.*, 2016). Entre ellos destacan Hierro (Fe), cinc (Zn) y manganeso (Mn), objeto de esta Tesis. Estos micronutrientes se consideran esenciales porque participan como cofactores de enzimas involucradas en varios procesos biológicos de las plantas, las funciones que desempeñan dentro de la planta no pueden ser remplazadas por otro elemento y la planta es incapaz de completar su ciclo de vida en ausencia de estos elementos (Marschner, 2012; Kumar *y col.*, 2016).

1.1.1 Hierro

El Fe es el cuarto elemento más abundante en la corteza terrestre después del oxígeno, silicio y aluminio. Puede existir en dos estados de oxidación, Fe^{2+} y Fe^{3+}. En las plantas, el Fe es preferentemente absorbido en su forma divalente, pero esto depende el tipo de estrategia (I o II) que presenten las plantas (sección 1.1.1.1.1) (Marschner, 2012). En la disolución del suelo el Fe(III) puede estar presente formando diversas especies hidrolizadas dependiendo del pH. De acuerdo con Lindsay (1988), el $Fe(OH)_2^+$ es la especie dominante por debajo de un pH de 7.4, el $Fe(OH)_4^-$ por encima de un pH de 8.5 y el $Fe(OH)_3^\circ$ predomina en el intervalo de 7.4-8.5. En este intervalo de pH, la solubilidad del Fe se encuentra por debajo de la concentración óptima para las plantas ($10^{-7.7}$ M) (Lindsay y Schwab, 1982). Es por ello que la disponibilidad del Fe para la planta en suelos calizos está limitada por el alto contenido de $CaCO_3$ que mantiene el pH entre 7.5-8.5.

1.1.1.1 Síntomas de deficiencia de Fe

La concentración crítica de deficiencia de Fe en hoja, varía entre una especie y otra e incluso entre los cultivares, la cual se encuentran dentro del intervalo de 50-100 mg Kg^{-1} peso seco (Barker y Pilbeam, 2007; Marschner, 2012). La deficiencia de este micronutriente ha mostrado una mayor susceptibilidad en cultivos como soja (*Glycine max*), judía (*Phaseolus vulgaris*), vid (*Vitis vinífera*), tomate (*Solanum lycopersicum* L.) y fresa (*Fragaria*) (Lucena *y col.*, 2008; Carrasco-Gil *y col.*, 2016; Gama *y col.*, 2016; Martín-Fernández *y col.*, 2017). De acuerdo con Tomasi *y col.* (2009), la deficiencia de Fe se caracteriza por una clorosis intervenal en las hojas (Figura I.1) e incrementos en el diámetro de la zona sub-apical y la relación raíz:parte aérea, así como un incremento en la proliferación de raíces laterales para permitir una mayor absorción (Gama *y col.*, 2016). La clorosis férrica es consecuencia de una disminución en la

concentración de la clorofila, dado que el Fe es requerido en la síntesis del ácido δ-aminolevulínico y la protoclorofilida, los cuales son precursores de la clorofila (Marschner, 2012).

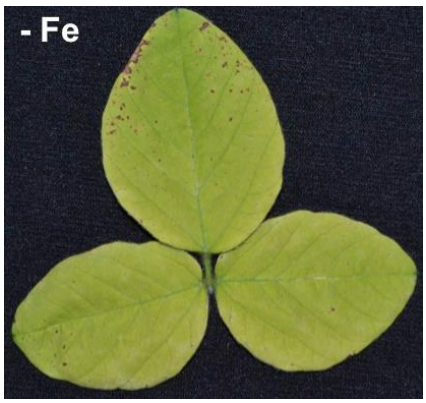

Figura I.1. Síntomas de deficiencia de Fe en soja. Fuente: Propia.

1.1.1.1.1 Estrategias de respuesta a la deficiencia de Fe

Las dos estrategias de absorción de Fe desarrollado por las plantas, son el principal factor para afrontar su deficiencia cuando crecen en suelos calizos. La estrategia I, también conocida como de reducción, se activa en dicotiledóneas y monocotiledóneas no-gramíneas. Esta estrategia consiste en la liberación de ácidos orgánicos, cumarinas y riboflavinas a través de sus raíces, resultando, junto con la excreción de H^+ promovido por la enzima H+-ATPasa de la membrana plasmática (AHA2), en un incremento de la solubilidad del Fe(III) (Chen *y col*., 2017; Connorton *y col*., 2017). Además, el desarrollo de raíces laterales es mediado por la enzima H+-ATPasa (AHA7) en condiciones de deficiencia de Fe (Santi y Schmidt, 2009). Posteriormente, el Fe(III) es reducido a Fe^{2+} por la enzima Fe quelato reductasa (FRO2) en la membrana plasmática, como un paso previo a su absorción dentro de la raíz a través del transportador férrico (ITR1). Las plantas gramíneas como cebada, maíz, trigo y arroz activan una estrategia basada en la quelación de micronutrientes conocida como estrategia II. Estas plantas excretan fitosideróforos (aminoácidos no proteicos), pertenecientes a la familia de los ácidos mugineicos, los cuales son sintetizados a partir del compuesto S-adenosilmetionina que es transformado en nicotinamina y finalmente en ácido mugineico, y que es excretado mediante el transportador de ácido mugineico TOM1. Estos fitosideróforos solubilizan el Fe en la rizósfera, a partir de la formación de complejos Fe-fitosideróforo como paso previo a su absorción mediante un transportador especifico (YS1) (Chen *y col*., 2017; Connorton *y col*., 2017). Ambas estrategias han permitido establecer un factor clave en las bases para el mejoramiento

de plantas susceptibles y mejores prácticas agronómicas en el uso de fertilizantes para corregir las deficiencias de micronutrientes (Lucena y Hernández-Apaolaza, 2017).

1.1.2 Manganeso

El Mn es el doceavo elemento más abundante en la superficie terrestre (Barker y Pilbeam, 2007). Aunque el Mn puede existir en diferentes estados de oxidación (I, II, III, IV, VI y VII), en sistemas biológicos prevalecen el II, III y IV de los cuales el III es más inestable (Marschner, 2012). La absorción de Mn por las plantas tiene lugar a través de la forma Mn^{2+} y es mediada por la reducción de óxidos de Mn a partir de tres estrategias combinadas de reducción: **1)** la reducción enzimática en la superficie de la raíz, **2)** la exudación de compuestos fenólicos y carboxilatos como el malato, y **3)** la actividad de microorganismos reductores de Mn en la rizósfera. El reemplazamiento del Mn^{2+} (0.075 nm) por otros cationes de similar radio iónico como el Mg^{2+} (0.065 nm), Ca^{2+} (0.099 nm) y Zn^{2+} (0.069 nm) puede limitar la absorción del Mn^{2+} por la planta (Marschner, 1988). Sin embargo, la oxidación del Mn(II) a Mn(III) o Mn(IV) en condiciones de suelo calizo también es un factor limitante en la disponibilidad del Mn debido a la formación de precipitados como manganita (γ-MnOOH) y pirolusita (β-MnO_2) y diversos fosfatos (Norvell, 1988). Además, en condiciones reductoras puede formarse la rodocrosita ($MnCO_3$) (Schwab y Lindsay, 1983).

1.1.2.1 Síntomas de deficiencia de Mn

Los cultivos como soja (*Glycine max*), avena (*Avena sativa* L.), trigo (*Triticum aestivum* L.) y melocotón (*Prunus persica* L) son más susceptibles a la deficiencia de Mn (Marschner, 2012; Martín-Ortiz *y col.*, 2016). La concentración crítica de deficiencia se encuentra dentro del intervalo de 10-20 mg kg^{-1} peso seco (Barker y Pilbeam, 2007; Marschner, 2012). Esta deficiencia se caracteriza por una inhibición en el desarrollo de la parte aérea (PA), el tamaño de la hoja y la floración debido al acortamiento de la raíz e inhibición del desarrollo de raíces laterales, así como una clorosis intervenal que se extiende entre las venas laterales de hojas jóvenes (Figura I.2) y la aparición de puntos necróticos en condiciones de extrema deficiencia (Barker y Pilbeam, 2007; Marschner, 2012). El acortamiento en el crecimiento de la raíz es causado por la disminución en la concentración de carbohidratos en la raíz, ya que el Mn participa como cofactor de la enzima fosfoenolpiruvato carboxiquinasa en el ciclo de los ácidos tricarboxílicos (Marschner, 2012). Los síntomas de clorosis intervenal son el resultado de daños en la estructura de los tilacoides y del fotosistema II que promueven la degradación de clorofila (Simpson y Robinson, 1984). Los daños causados en la

estructura de los tilacoides son causados por la inhibición en la biosíntesis de lípidos y carotenoides (Marschner, 2012).

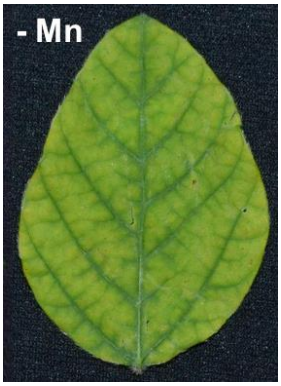

Figura I.2. Síntomas de deficiencia de Mn en soja. Fuente: Propia.

1.1.3 Cinc

El Zn es el vigésimo cuarto elemento más abundante en la superficie terrestre, existiendo en forma divalente, exclusivamente, ya que no participa en reacciones redox (Marschner, 2012). En las plantas, el Zn es preferencialmente absorbido como Zn^{2+} por contacto directo con la raíz, pero a alto pH puede ser absorbido como $ZnOH^{+}$ (Barker y Pilbeam, 2007). La concentración del Zn en suelos cultivados es alrededor de 65 mg kg^{-1}, pero como en el caso del Fe y el Mn, la solubilidad del Zn en suelos calizos también es reducida por la formación de óxidos, ferrita o fosfatos insolubles. La smithsonita ($ZnCO_3$) formada a altos pHs es algo más soluble (Alloway, 2008).

1.1.3.1 Síntomas de deficiencia de Zn

La concentración crítica de Zn en hoja varía entre los cultivos sensibles (judía, maíz, arroz, manzanas y cítricos). La deficiencia ocurre principalmente dentro del intervalo de 15-20 mg kg^{-1} peso seco (Barker y Pilbeam, 2007; Marschner, 2012). De acuerdo con Marschner (2012), la deficiencia de Zn se caracteriza por un crecimiento inhibido de la planta y el desarrollo de hojas pequeñas, lo cual está relacionado con un descenso en la producción de auxinas, como el ácido indolacético (AIA), y las giberelinas. En condiciones de severa deficiencia, se puede observar una clorosis difusa con la aparición de puntos necróticos en las hojas más viejas, causada por la toxicidad de otros elementos. Esto es consecuencia del incremento en la permeabilidad de la membrana plasmática. A su vez, el daño causado en la membrana plasmática es debido al aumento en la producción de especies reactivas de

oxígeno, como resultado del descenso en la actividad de la enzima CuZn superóxido dismutasa por la inhibición en la síntesis de proteínas.

1.2 Factores que influyen en la deficiencia de micronutrientes

Un gran número de factores del suelo y de la planta afectan a la absorción de micronutrientes por las plantas (Barker y Pilbeam, 2007). Entre los factores del suelo que intervienen negativamente en la absorción y rendimiento de los cultivos son:

1) La textura del suelo; es un factor importante en la lixiviación o mantenimiento de micronutrientes. De acuerdo con la recopilación de Kumar *y col*. (2016), los suelos arenosos son más deficientes en micronutrientes en comparación con los suelos arcillosos, como consecuencia de la elevada lixiviación causada por su baja capacidad para mantener agua. No obstante, un alto contenido de arcilla también puede reducir la disponibilidad de micronutrientes debido a la adsorción de los micronutrientes, como ha sido observado por Almendros *y col*. (2013), en tiestos de suelo fertilizados con Zn:aminoLS, Zn:EDDS y Zn:polihidroxifenilcarboxilato.

2) Las bajas temperaturas del suelo; influyen en la difusión de los micronutrientes, debido a que retrasan la respuesta a las deficiencias de micronutrientes por la inhibición de la actividad de la raíz (Barker y Pilbeam, 2007; Alloway, 2008).

3) El contenido de humedad; en suelos calizos inundados o al 60 % de capacidad de campo, se reduce la disponibilidad de micronutrientes a largo plazo, debido a la reducción en el potencial redox, permitiendo solo concentraciones de micronutrientes disponibles a corto plazo para complejos estables como fue observado para el Zn:EDDS por Almendros *y col*. (2013). Además, un bajo contenido de agua en el suelo, también puede reducir la disponibilidad de micronutrientes, como consecuencia de la disminución de crecimiento de raíces (Marschner, 2012).

4) La interacción entre micronutrientes en el sistema planta-suelo; este también es un factor importante en la absorción de micronutrientes por las plantas. Kumar *y col*. (2016) han recopilado interacciones antagónicas entre Fe, Mn y Zn, así como entre Cu y Zn

5) El bajo contenido de materia orgánica; juega un rol importante, ya que influye en el contenido de transportadores, como las sustancias húmicas, que participan en la solubilización de metales.

6) Elevadas concentraciones de bicarbonato o $CaCO_3$ típico de suelos calizos; incrementan el pH del suelo y lo tamponan. El alto pH influye en la deficiencia de micronutrientes, como consecuencia de la formación de hidróxidos y carbonatos (Lucena, 2000; Almendros *y col.*, 2013). De acuerdo con Barker y Pilbeam (2007) más del 30% de suelos cultivables a nivel mundial son alcalinos (Figura I.3).

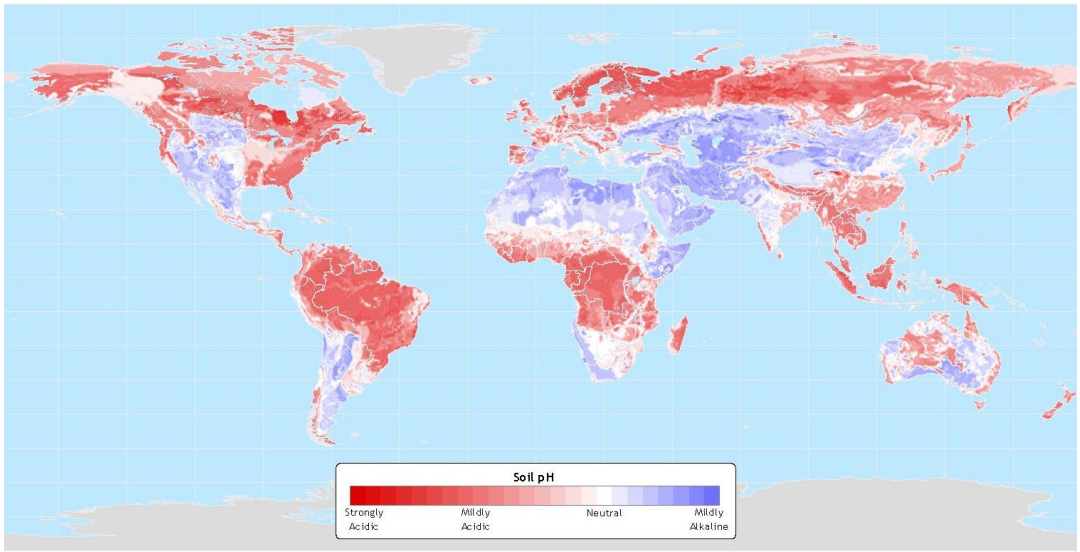

Figura I.3. Suelos alcalinos a nivel mundial. Fuente: IGBP-DIS, 1998.

1.3 Estrategias para corregir las deficiencias de micronutrientes metálicos en plantas

A través de los años se han empleado distintas estrategias para afrontar las deficiencias de micronutrientes en los cultivos y/o mejorar la disponibilidad de micronutrientes en el sistema suelo-planta. Estas incluyen la selección varietal y la adición de fertilizantes en cultivos ya establecidos (Lucena y Hernández-Apaolaza, 2017) que se reserva para variedades no eficientes o situaciones de carencia del elemento en cuestión. A parte de la selección varietal tradicional, se está realizando el mejoramiento genético a partir de diversas herramientas moleculares para el desarrollo de nuevos cultivos tolerantes a la deficiencia de micronutrientes (Aksoy *y col.*, 2017).

1.3.1 Mejoramiento de cultivares más resistentes

Investigaciones para elucidar los mecanismos moleculares involucrados en la expresión de genes relacionados con las deficiencias de micronutrientes. Para ello se usan plantas modelo como *Arabidopsis* o la soja, la cual además es un cultivo de gran importancia agronómica (Aksoy *y col.*, 2017). Así, Nozoye *y col.* (2014) demostraron

que plantas de soja modificadas transgénicamente para la sobreexpresión el gen *HvNAS1* incrementaron la producción de nicotinamina, y, como consecuencia, la absorción de Fe y Zn en condiciones de suelo calizo, en comparación con plantas de soja no transgénicas. Además, el mejoramiento genético también ha permitido la biofortificación de diferentes cultivos, tales como soja, trigo y arroz para incrementar el contenido de Fe y su translocación en cultivos a partir de la sobreexpresión o silenciamiento de genes (Connorton *y col*., 2017).

1.3.2 Adición de fertilizantes

Los fertilizantes usados para corregir las deficiencia de micronutrientes metálicos son clasificados en tres principales grupos: sales inorgánicas, quelatos sintéticos y complejos naturales (EU, 2003). De acuerdo con la regulación oficial europea de fertilizantes (EU, 2003), sólo podrán ser considerados como fertilizantes, los productos que aporten nutrientes a las plantas de una manera eficaz, no produzcan efectos nocivos a la salud y al medioambiente, así como que se disponga de métodos adecuados para comprobar su contenido. Aunque los requisitos de la legislación del 2003 siguen vigentes, ésta ya ha sido reemplazada por la nueva legislación del 2019 (EU, 2019). Además, ésta nueva legislación establece que los productos deberán permanecer estables al menos 1 día en solución acuosa a pH 6-7 y al menos 3 días en disolución Hoagland a pH 7-8 para poder ser considerados fertilizantes (EU, 2019). No obstante, la normativa española (BOE, 2017) amplia la lista de los productos permitidos para su uso como fertilizantes en España, incluyendo los agentes complejantes descritos más adelante en la sección 1.2.2.3.

1.3.2.1 Sales inorgánicas

Los micronutrientes tales como Fe, Mn y Zn pueden ser aplicados como sales solubles (mayormente sulfatos) a los cultivos. Sin embargo, la formación de hidróxidos o óxidos insolubles bajo condiciones de suelo calizo (pH 7.4-8.5) implica una reducción en su solubilidad (Martín-Ortiz *y col*., 2009; Shaddox *y col*., 2016). De acuerdo con Lindsay and Schwab (1982), cuando una sal de Fe(III) o Fe(II) son adicionadas en un suelo con un intervalo de pH entre 7.4 y 8.5, ésta precipita rápidamente en forma de ferrihidrita (1-3 días). Esta especie presentan una limitada solubilidad en el intervalo de pH de suelos calizos, siendo necesario adicionar formas más solubles de Fe para contrarrestar la deficiencia de Fe en plantas. En el caso de la adición de $ZnSO_4$ en suelos calizos, este reacciona con el $CaCO_3$ induciendo la precipitación del Zn en forma de minerales insolubles y/o $ZnCO_3$ (smithsonita) (Marschner, 2012). En el caso del Mn, la solubilidad de este micronutriente está limita a elevado pH y medio aireado,

ya que se produce la formación de MnO_2 (pirolusita), que es muy insoluble (Norvell, 1988). Así, aunque Goos y Germain (2001) han sugerido que la aplicación de altas dosis de micronutrientes a la semilla puede aliviar las deficiencias causadas por su baja solubilidad, la aplicación de quelatos en cultivos crecidos en suelos calizos es hasta ahora la mejor recomendación para evitar la precipitación de micronutrientes a partir de sales como son los sulfatos (Lucena, 2006).

1.3.2.2 Quelatos sintéticos

Los agentes quelantes son compuestos, principalmente poliaminocarboxílicos, capaces de mantener los micronutrientes solubles, de modo que evitan la precipitación del metal en forma de hidróxidos insolubles debido a la alta estabilidad del quelato formado (Lucena, 2009). Los quelatos usados como fertilizantes se clasifican en fenólicos y no fenólicos, dependiendo de la presencia de este grupo funcional en su estructura (Figura I.4). El Reglamento Europeo 1618/2016 (EU, 2016) actualización del reglamento de productos fertilizantes 2003/2003 (EU, 2003) establece los siguientes productos fenólicos o ácidos poliaminofenolcarboxílicos para su uso como quelantes en fertilizantes de micronutrientes: ácido etilendiamino-di-(*o*-hidroxifenilacético) (*o,o*-EDDHA), ácido etilendiamino-N-(*o*-hidroxifenilacético)-N'-(*p*-hidroxifenilacético) (*o,p*-EDDHA), ácido etilendiamino-N'N-di-(o-hidroximetilfenilacético) (*o,o*-EDDHMA), ácido etilendiamino-di-(2-hidroxi-5-sulfofenilaácetico) (EDDHSA) y ácido N,N'-bis (*o*-hidroxibenzil) etilendiamino-N,N'-diacético (HBED). El *o,p*-EDDHA presenta una estabilidad menor que el *o,o*-EDDHA, como consecuencia de que sólo cinco de sus seis grupos donadores son capaces de unirse al metal (Yunta *y col*., 2003). Al contrario, el HBED presenta una alta estabilidad en suelo calizo similar al *o,o*-EDDHA debido a su mayor afinidad por el Fe (López-rayo *y col*. 2009; Martín-Fernández *y col*., 2017). En el caso de los productos no fenólicos o ácidos poliamino carboxílicos se encuentran: ácido etilendiaminotetracético (EDTA), ácido dietilentriaminopentaácetico (DTPA), ácido 2-hidroxietiletilendiaminotriacético (HEEDTA), ácido etilendiaminosuccínico [S,S]-EDDS y el ácido iminodisuccínico (IDHA). El [S,S]-EDDS y el IDHA presentan una estructura análoga al EDTA con seis y cinco grupos donadores, respectivamente. Si bien en suelo ambos ligandos presentan una menor estabilidad que el EDTA, su biodegradabilidad los hace una alternativa ambientalmente sostenible a los ligandos tradicionales para proveer micronutrientes en hidroponía (Lucena *y col*., 2008; Rodríguez-Lucena *y col*., 2010) o suelo calizo (López-Rayo *y col*., 2015, 2019).

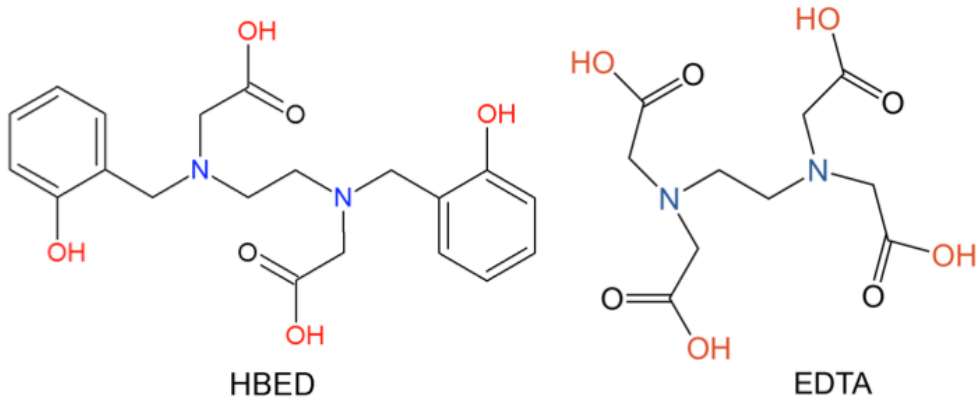

Figura I.4. Ejemplo de agentes quelante fenólicos (HBED) y no fenólicos (EDTA).

La aplicación de estos productos es la práctica más común para prevenir o remediar la deficiencia de micronutrientes metálicos en cultivos de alto valor económico (Lucena, 2006). Para Fe, los agentes quelantes más usados son los fenólicos como EDDHA, ya que presenta una mayor estabilidad para metales trivalentes, como el Fe^{3+}, que divalentes, como el Mn^{2+} o Zn^{2+}, debido a que su estructura consiste principalmente de bases fuertes de Lewis (Schenkeveld *y col.*, 2014). Este agente quelante presenta dos isómeros posicionales: el *o,p*-EDDHA que es más efectivo a corto plazo para corregir plantas deficientes bajo condiciones de hidroponía (García-Marco *y col.*, 2006; López-Rayo *y col.*, 2012) y el *o,o*-EDDHA, el cual es ampliamente usado como fertilizante en condiciones de suelo calizo. El HBED es similar al *o,o*-EDDHA y muestra una mayor capacidad de translocación de Fe, así como un efecto a largo plazo en suelo calizo (Martín-Fernández *y col.*, 2017a). Lindsay y Schwab (1982) también demostraron que el *o,o*-EDDHA presenta una mayor estabilidad con Fe en comparación con EDTA, que es estable hasta un pH de 6.7, y en comparación con DTPA, que es estable hasta un pH de 7.7.

Los agentes quelantes como EDTA, HEEDTA, DTPA, S,S-EDDS y *o,p*-EDDHA han mostrado ser eficaces para mantener el Mn soluble a pH 8, mientras que a un pH de 9 el EDTA y el EDDS han mostrado una mayor capacidad. El EDTA y el DTPA también han mostrado capacidad para proveer Mn en condiciones de suelo calizo (Boxma y de Groot, 1971; López-Rayo *y col*, 2014, 2019). Sin embargo, en experimentos de incubaciones en suelo en un intervalo de pH de 7.3-7.8, los agentes quelantes *o,p*-EDDHA, IDHA y S,S-EDDS han mostrado una baja capacidad para mantener soluble el Mn en comparación con los ligandos tradicionales como el EDTA, DTPA y HEEDTA (López-Rayo *y col.*, 2015).

En el caso del Zn, el EDTA es el agente quelante más comúnmente usado, ya que presenta una alta afinidad por este micronutriente, mayor que por el Ca^{2+}, lo que le confiere una mayor estabilidad en condiciones de hidroponía y suelo calizo.(Álvarez y Rico, 2003; Alloway, 2008). Otros agentes quelantes que también han mostrado ser eficaces en solución alcalina para mantener el Zn soluble son el S,S-EDDS, DTPA, IDHA y *o,p*-EDDHA, los cuales son estables a un pH hasta 9.0 (Lucena *y col.*, 2008; López-Rayo *y col.*, 2012). Por el contrario, López-Rayo *y col.* (2015), demostraron que los agentes quelantes IDHA y *o,p*-EDDHA tienen una menor capacidad que DTPA, EDTA, HEEDTA y S,S-EDDS para mantener el Zn soluble en suelo en un intervalo de pH 7.3 y 7.8.

De acuerdo con Lucena (2009) la eficiencia de los quelatos para proveer micronutrientes a la planta cuando son aplicados al suelo calizo depende de tres parámetros:

1) La capacidad del agente quelante para solubilizar micronutrientes de la fase sólida después de haber cedido su metal a la planta, conocido como efecto de recarga o "shuttle effect".

2) La reactividad del agente quelante liberado o del propio quelato, la cual influye en su retención por las superficies de intercambio del suelo o del quelato. Hernández-Apaolaza y Lucena (2011), mostraron que el *o,o*-EDDHA:Fe^{3+} presentaba retención en columnas de lixiviación con suelo calizo. Asimismo, Martín-Fernández *y col.* (2017b) demostraron que el *o,p*-EDDHA:Fe^{3+} y meso *o,o*-EDDHA:Fe^{3+} presentaban mayor interacción que el HBED:Fe^{3+} en ensayos de interacción con suelo calizo, turba y ferrihididrita.

3) Las reacciones de competencia por otros metales presentes en el medio, cuando el agente quelante es liberado. Así, López-Rayo *y col.* (2019) demostró que el Fe complejado por EDTA puede ser desplazado por Mn en condiciones de suelo calizo, a partir de una disminución en la relación molar Fe/Mn en hoja. También, Martín-Fernández *y col.* (2017b) observaron desplazamiento de Fe por Cu en una mezcla de EDDHA:Fe^{3+} aplicada en suelo calizo, lo cual fue atribuido principalmente a la afinidad de Cu con el *o,p*-EDDHA y meso *o,o*-EDDHA. Por el contrario, el *o,o*-EDDHA ha mostrado un mayor desplazamiento de Mn y Zn por Fe (López-Rayo *y col.*, 2015).

A pesar de la alta eficiencia de los quelatos sintéticos, su escasa biodegradabilidad y el incremento de riesgo de lixiviación de metales en condiciones de campo ha llevado a aplicar nuevos quelatos sintéticos más biodegradables como el IDHA y el [S,S]-EDDS, los cuales han mostrado proveer micronutrientes bajo condiciones de

hidroponía, fertirrigación y suelos calizos (Lucena *y col.*, 2008; Rodríguez-Lucena *y col.*, 2010; López-Rayo *y col.*, 2012, 2019). No obstante, su efectividad a largo plazo puede ser reducida como consecuencia de las reacciones del suelo que aceleran su biodegradabilidad o de la competición con otros metales, como ha sido demostrado por (López-Rayo *y col.*, 2015) para IDHA y [S,S]-EDDS aplicados en suelo calizo. Por tanto es necesario incrementar la frecuencia de aplicación y dosis. De igual manera, el elevado costo de los quelatos sintéticos también ha influido en la búsqueda de otras alternativas biodegradables y más económicas como los agentes complejantes.

1.3.2.3 Agentes complejantes

Una alternativa más económica para mejorar la fertilización de micronutrientes metálicos y reemplazar los quelatos sintéticos, es el uso de productos con menor impacto ambiental y mayor biodegradabilidad (Rodríguez-Lucena *y col.*, 2010). Estos productos incluyen especies poliméricas como humatos y LS y no poliméricas como ácidos orgánicos, aminoácidos, G6 y G7, incluidos en la lista de complejos autorizados según el Real Decreto 999/2017 (BOE, 2017).

1.3.2.3.1 Ácidos orgánicos

Los ácidos orgánicos son compuestos de bajo peso molecular que pueden ser de origen natural o sintético. Se ha observado la excreción de ácidos orgánicos como citrato, malato y ascorbato de forma natural en plantas de estrategia I y II deficientes de Fe (Abadía *y col.*, 2002). Sin embargo, la síntesis de estos productos a gran escala se realiza mediante la fermentación de glucosa por el hongo *Aspergillus niger*, el cual secreta varios ácidos orgánicos incluyendo el citrato (Odoni *y col.*, 2017).

Estudios con ácidos orgánicos, especialmente citrato, han demostrado que tienen una capacidad efectiva para complejar Fe. Sin embargo, su habilidad para proveerlo a la planta es más baja que la de los quelatos sintéticos en condiciones de hidroponía o aplicación foliar (Rodríguez-Lucena *y col.*, 2010). Kovács *y col.* (2016) también demostraron una baja estabilidad de Fe-citrato aplicado a plantas deficientes de pepino en condiciones de hidroponía, observando una acumulación de Fe en la raíz que puede ser utilizada en la biofortificación de Fe. Por el contrario, una mezcla de citrato, sorbitol y malato con sulfato ferroso mostró un incremento de clorofila en hojas cloróticas de kiwi (Rombolà *y col.*, 2002). Estos resultados son debidos a que diferentes parámetros como el pH de la solución del suelo, el tipo de metal y el número y proximidad de ácidos carboxílicos presentes en la molécula influyen en su capacidad complejante. Jones (1998) concluyó que los ácidos monocarboxílicos presentaron más

baja capacidad para complejar Fe^{3+} que los tricarboxílicos (malato, citrato y oxalato). Sin embargo, su capacidad de complejación con Fe^{3+} o Mn^{2+} disminuyó con el incremento del pH en el suelo o en presencia de Ca^{2+}.

1.3.2.3.2 Aminoácidos

Los aminoácidos libres son productos de origen natural conformados por grupos funcionales amino, carboxilo y en algunas ocasiones nitrógeno heterocíclico, fenol y sulfhidrilo con capacidad para complejar metales (Lucena, 2009).

De acuerdo con Souri (2016), los complejos obtenidos a partir de aminoácidos deben cumplir tres parámetros: 1) un peso molecular de 150 a 200 Da que les permita una mejor absorción por las hojas o la raíz, 2) carga neutra para evitar interacciones con otros compuestos previo a su absorción y 3) ser altamente solubles en agua y disponibles para que puedan ser metabolizados dentro de la planta. Diversos autores han demostrado que los aminoácidos son capaces de proveer micronutrientes a diferentes cultivos por aplicación al suelo o vía foliar. Por ejemplo, Ghasemi *y col*. (2013) demostraron la efectividad de arginina, histidina y glicina para mejorar la biodisponibilidad de Zn y el rendimiento del trigo por aplicación foliar en comparación con sulfato de zinc. Estos aminoácidos también presentan una mejor absorción y translocación de Fe y Zn en plantas de tomate bajo condiciones de hidroponía en comparación con Fe-EDTA (Ghasemi *y col*., 2012). Asimismo, Rafie *y col*. (2017) observaron mejor rendimiento de cebollas crecidas en suelos calizos cuando se les aplicaba un complejo Zn-lisina en comparación con el sulfato de zinc Por el contrario, glicina, arginina y glutamato presentan baja eficiencia para proveer Fe a plantas de soja bajo condiciones de hidroponía (Rodríguez-Lucena *y col*., 2010). Sin embargo, García *y col*. (2011) confirmaron que la absorción de micronutrientes en disolución nutritiva depende del tipo de aminoácido, de modo que aminoácidos alifáticos (serina y alanina) incrementaron la concentración de Fe, Mn, Cu y clorofila en plantas de tomate. Li *y col*. (2017) recopilaron resultados similares, la serina presentó una mayor capacidad para complejar Fe^{3+} debido a la presencia de su grupo hidroxilo mientras el Zn es mejor complejado por la cisteína debido a su grupo sulfhidrilo.

1.3.2.3.3 Humatos

Las sustancias húmicas son macromoléculas de estructura variada procedentes de suelos, turba, leonardita y derivados de sistemas acuáticos (Lucena, 2009).

Estos productos también son agentes complejantes efectivos debido a la coordinación del metal con grupos carboxílicos, fenólicos y/o hidroxilos (Kovács *y col.*, 2013). Además, se ha comprobado su efectividad como correctores nutricionales en cítricos crecidos en suelos calizos (Sánchez-Sánchez *y col.*, 2002; Cieschi *y col.*, 2017). También, Kovács *y col.* (2013) sugirieron que los complejos de Fe-leonardita pueden ser usados en condiciones de suelo calizo, tras demostrar su eficiencia bajo condiciones hidropónicas eficazmente. Chen *y col.* (2004) obtuvieron un resultado similar en plantas de soja crecidas en hidroponía suplidas con ácidos húmicos y fúlvicos procedentes de leonardita, resultando en un incremento en la clorofila y movilización de Fe y Zn dentro de las plantas. Ozkutlu *y col.* (2006) también confirmaron la efectividad de humatos de Zn para corregir plantas de soja deficientes de Zn en suelos calizos. Asimismo, Pérez-Sanz *y col.* (2006) sugirieron que se pueden utilizar fracciones de alto y bajo peso molecular obtenidas a partir de complejos de Fe con humatos como fertilizantes de lenta y rápida liberación de Fe, respectivamente. Este resultado ha sido confirmado por Tomasi *y col.* (2013), donde fracciones de bajo peso molecular de sustancias húmicas mostraron una mayor efectividad para proveer Fe bajo condiciones hidropónicas comparado con otros complejos como citrato o fitosideróforos. Cieschi *y col.* (2017) ha demostrado una liberación lenta del Fe a partir de un complejo de Fe con leonardita cuando se aplicó en arboles de cítricos crecidos en suelo calizo.

Por el contrario, Rodríguez-Lucena *y col.* (2010) mostraron que, aunque los humatos tienen una alta capacidad para complejar Fe, estos complejos no fueron capaces de incrementar la concentración de Fe en hojas de soja bajo condiciones de hidroponía. Sin embargo, al igual que en el resto de agentes complejantes, existen diferentes factores que pueden influir en su capacidad de complejación como son: pH, peso molecular, contenido de grupos funcionales y el metal complejado. De acuerdo con Chen y Stevenson (1986), los metales trivalentes se unen más fuertemente en comparación con los metales divalentes, independientemente del pH o la fuerza iónica.

1.3.2.3.4 Lignosulfonatos

Los lignosulfonatos (LS, Figura I.5) son subproductos de la industria del papel obtenidos a partir de maderas blandas (pino y abeto) o duras (eucalipto), los cuales son producidos a partir del proceso al sulfito (Lin y Dence, 1992). Este proceso consiste en la digestión la lignina entre 140-170ºC con sales de sulfito o bisulfito de sodio, amonio, magnesio, calcio o potasio (Calvo-Flores *y col.*, 2015).

Estos agentes complejantes han sido incluidos en la directiva de fertilizantes 223/2012 (EU, 2012) que actualizó la directiva de fertilizantes 2003/2003 (EU, 2003). La capacidad de los LS para complejar micronutrientes metálicos es promovida por la amplia variedad de grupos funcionales presentes en su estructura (Figura I.5), tales como grupos fenólicos, hidroxílicos, sulfónicos y carboxílicos (Pang *y col.*, 2008; Rodríguez-Lucena *y col.*, 2011). El contenido de grupos funcionales depende del proceso de modificación aplicado a los LS, tales como la hidroximetilación, fenolación, sulfonación y oxidación (Aro y Fatehi, 2017). Igualmente se puede influir en el peso molecular, usando la ultrafiltración a partir de la cual se obtienen LS de bajo peso molecular (Benedicto *y col.*, 2011). Además, la fuente de madera a partir de la cual son extraídos los LS también puede influir en los grupos funcionales y el peso molecular (Meier *y col.*, 1994; Myrvold, 2016).

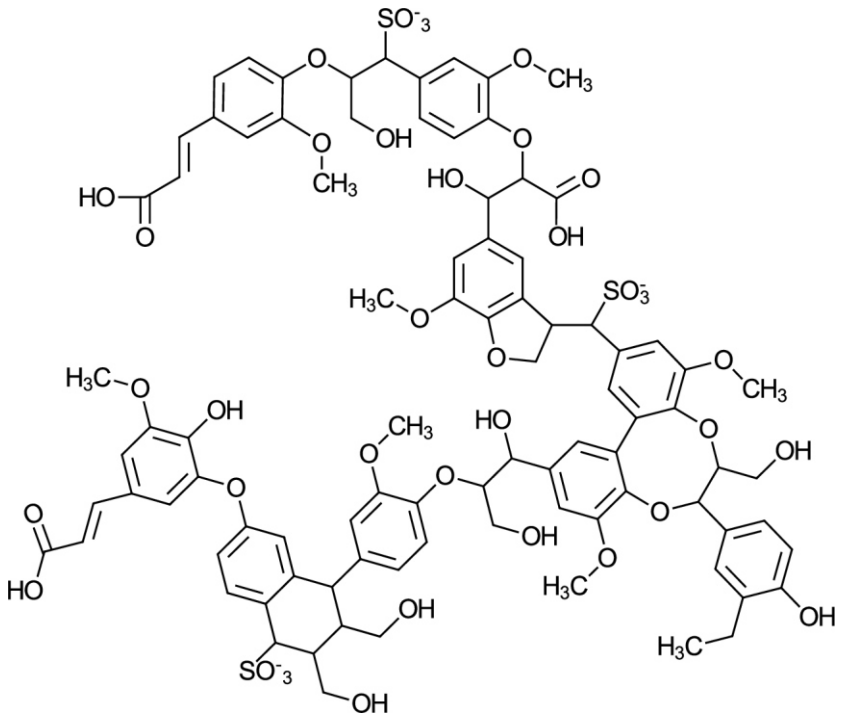

Figura I.5. Estructura de lignosulfonato. Modificado de: Kun y Pukánszky, 2017.

Con respecto a la efectividad de los LS como fertilizantes, distintos autores confirmaron que pueden ser una fuente de Zn eficiente en plantas de judía y maíz, ya sea aplicado a la hoja o en condiciones de hidroponía o suelo calizo (Martín-Ortiz *y col.*, 2009; Benedicto *y col.*, 2011; Cieschi *y col.*, 2016; Minnocci *y col.*, 2018). Otros investigadores también demostraron la efectividad de LS con Fe aplicados foliarmente o en suelo calizo (Rodríguez-Lucena *y col.*, 2009, 2011; Martín-Fernández *y col.*, 2017a) para cultivos como pepino, tomate y soja. Finalmente, López-Rayo *y col.* (2014) confirmaron la capacidad de los LS para mantener el Mn soluble a partir de

experimentos de interacción con suelos. Asimismo, Martín-Ortiz *y col*. (2016) demostraron la eficiencia de los LS como fertilizante de Mn en condiciones de hidroponía y suelo calizo para trigo

1.3.2.3.5 Gluconatos

El ácido glucónico (Figura I.6) es un agente complejante biodegradable obtenido a partir de la fermentación aerobia de glucosa con *Aspergillus niger* a pH 6.0-6.5 y a 34ºC, comercializado principalmente en su sal sódica a partir de la adición de NaOH durante el proceso de fermentación (Ramachandran *y col*., 2017).

La presencia de grupos hidroxílicos y carboxílicos en la estructura de gluconatos implica una alta capacidad para complejar Mn (López-Rayo *y col*., 2012) en soluciones alcalinas a pH 9. Por el contrario, también se ha demostrado una baja estabilidad para mantener el Fe y Zn soluble en soluciones alcalinas a un pH>8 (Lucena *y col*., 2010; Rodríguez-Lucena *y col*., 2010; López-Rayo *y col*., 2012). Además, en experimentos desarrollados en tiestos con suelos calizos, se ha demostrado que el gluconato (G6) tiene una baja capacidad para mantener Fe, Mn y Zn solubles (Clemens *y col*., 1990; Goos y Germain, 2001; López-Rayo *y col*., 2015). Por el contrario, Martín-Fernández *y col*., (2017a) demostraron la corrección de clorosis en soja después de la aplicación de Fe^{3+}:G6 en suelos calizos.

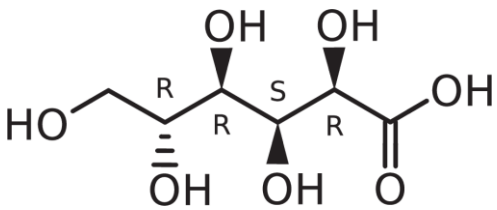

Figura I.6. Estructura del ácido glucónico. Fuente: Propia.

1.3.2.3.6 Heptagluconatos

El ácido heptaglucónico (Figura I.7) es un agente complejante producido principalmente en su sal sódica, el cual es obtenido a partir de la reacción de cianuro de sodio con glucosa (Maxwell, 2004). La reacción se realiza a 40ºC para eliminar el amonio formado, resultando en una mezcla de isómeros α y β (Zak, 1969).

Este agente complejante ha sido incluido en la directiva de fertilizantes 1618/2016 (EU, 2016). Su efectividad para complejar metales ha sido previamente demostrada por López-Rayo *y col*. (2014), donde el heptagluconato (G7) mostró una mayor

efectividad que el G6 para mantener el Mn disponible en soluciones alcalinas. Fuentes y col. (2018) también demostraron que el G7 fue tan eficiente como el EDTA en proveer Fe a plantas de pepino deficientes de Fe bajo condiciones de hidroponía, aunque la recuperación del índice de clorofila fue lento o la concentración de Fe metabólicamente útil para la planta baja. La efectividad de G7 como fertilizante en condiciones de suelo calizo fue estudiada por Álvarez y Rico (2003) que observaron que la aplicación de Zn-G7 mejoró el rendimiento y absorción de Zn en maíz (*Zea mays* L.) en comparación con la aplicación de Zn-LS. Además, Clemens *y col*. (1990) describieron que el G7 puede solubilizar más Fe que el G6 y EDTA en condiciones de suelo calizo. Sin embargo, otros estudios realizados en tiestos de suelos calizo mostraron una baja estabilidad de Fe y Mn complejados con G7 (Goos y Germain, 2001; Shaddox *y col*., 2016).

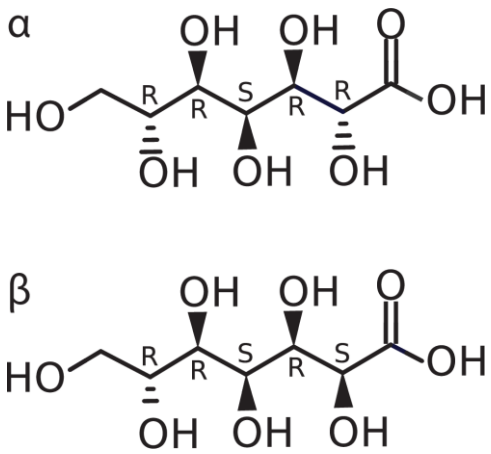

Figura I.7. Estructuras de los isómeros del ácido heptaglucónico. Fuente: Propia

1.4 Técnicas físico-químicas para la caracterización de ligandos y sus complejos

Para estudiar las características estructurales de los complejos, se vienen usando diferentes técnicas espectroscópicas que permiten determinar parámetros como el grado de pureza, geometría y estructura, así como los puntos de unión y el estado de oxidación de los metales.

1.4.1 Espectroscopía de Infrarrojos por transformada de Fourier

La espectroscopía de infrarrojos por transformada de Fourier (FTIR) ha sido usada para analizar los grupos funcionales de agentes complejantes (G6, humatos y LS) que participan en el proceso de complejación con Fe (Pérez-Sanz *y col*., 2006; Rodríguez-Lucena *y col*., 2011; Kovács *y col*., 2013; Nikolić *y col*., 2014). Por ejemplo, el análisis

de complejos de Mn y Zn por FTIR muestra la interacción con grupos hidróxilo y sulfonato (Gawluk *y col.*, 2017), mientras que para complejos de Fe, se observa, la participación de moléculas de agua (Nikolić *y col.*, 2014), grupos hidróxilo y fenol (Rodríguez-Lucena *y col.*, 2011). Esta técnica también ha sido usada para analizar fracciones de humatos de Fe, las cuales mostraron una diferencia en la capacidad de unión, siendo mayor para la fracción con mayor contenido de grupos carboxilato o fenolato que alifáticos (Pérez-Sanz *y col.*, 2006).

1.4.2 Resonancia magnética nuclear

La resonancia magnética nuclear (NMR) ha permitido determinar los grupos funcionales presentes en agentes complejantes de naturaleza polimérica tales como la leonardita (Kovács *y col.*, 2013). Asímismo, ha sido usada para analizar la pureza de agentes complejantes como ácido glucónico y heptaglucónico a partir de la comparación de señales correspondientes de cada espectro (López-Rayo *y col.*, 2014).

1.4.3 Espectroscopía de Mössbauer

La espectroscopia Mössbauer se ha utilizado para determinar la geometría de la coordinación de varios complejos de Fe a partir de humatos y fulvatos, LS, gluconatos, y azúcares, así como su estado de oxidación (Lakatos *y col.*, 1977; Tonković *y col.*, 1983; Carrasco *y col.*, 2012; Martín-Fernández *y col.*, 2017a). Esta técnica, no destructiva, también se ha aplicado para analizar las especies de Fe, tales como compuestos polinucleares y/o especies de oxihidróxidos de Fe presentes en las raíces de plantas de estrategia I (Kovács *y col.*, 2009; Martín-Fernández *y col.*, 2017a).

1.4.4 Cromatografía de filtración en gel

La cromatografía de exclusión por tamaño es una técnica para separar polímeros de acuerdo a su peso molecular, donde las moléculas de mayor tamaño eluyen primero de la columna, mientras que las partículas de bajo peso molecular penetran dentro de los poros de la columna, eluyendo al final (Figura I.8). Está técnica incluye, por una parte, la cromatografía de filtración en gel (GFC) sobre Sephadex® (gel formado por el entrecruzamiento de dextrano con epiclorhidrina) que es el material de empaque mayormente usado y por otra parte, la cromatografía de exclusión por tamaño de alta resolución (HPSEC) con mejor resolución cromatográfica y un análisis de tiempo más corto (Lin y Dence, 1992). Aunque estos métodos de cromatografía de exclusión por tamaño consisten en la separación por tamaño, también pueden influir otros efectos como la adsorción de solutos o el intercambio iónico en el comportamiento de las

muestras. Estas interferencias pueden ser suprimidas, de acuerdo con Chen y Li (2000), ajustando parámetros como el pH y la fuerza iónica.

1.4.5 Cromatografía de exclusión por tamaño de alta resolución

Desde los años 60 distintos autores han demostrado la efectividad de la técnica de GFC para determinar la distribución del peso molecular (MWD) de LS (Gupta y McCarthy, 1968; Ouyang *y col.,* 2011; Wang *y col*., 2012). Además ha sido aplicada para el análisis de quelatos, humatos y azucares con Fe (Boxema, 1979; Pérez-Sanz *y col*., 2006; Tonković *y col*., 1983) ligninas con Cu (Calabria y Gonçalves, 2006) y Zn complejado con péptidos (Xie *y col*., 2015). Igualmente, la técnica de HPSEC se ha usado para determinar la distribución del peso molecular de los LS procedentes de abeto y eucalipto (Fredheim *y col*., 2002; Braaten *y col*., 2003; Savy *y col*., 2018). Además, la combinación de HPSEC y cromatografía de par iónico se ha aplicado para distinguir LS que pueden ser usados para preparar formulaciones agroquímicas (Brudin *y col*., 2008). El uso de HPSEC acoplado con espectrometría de masas con plasma acoplado inductivamente (ICP) ha demostrado ser una herramienta útil para obtener evidencias de la unión del metal con materia orgánica disuelta procedente de aguas naturales (Worms *y col*., 2010; Wu *y col*., 2004).

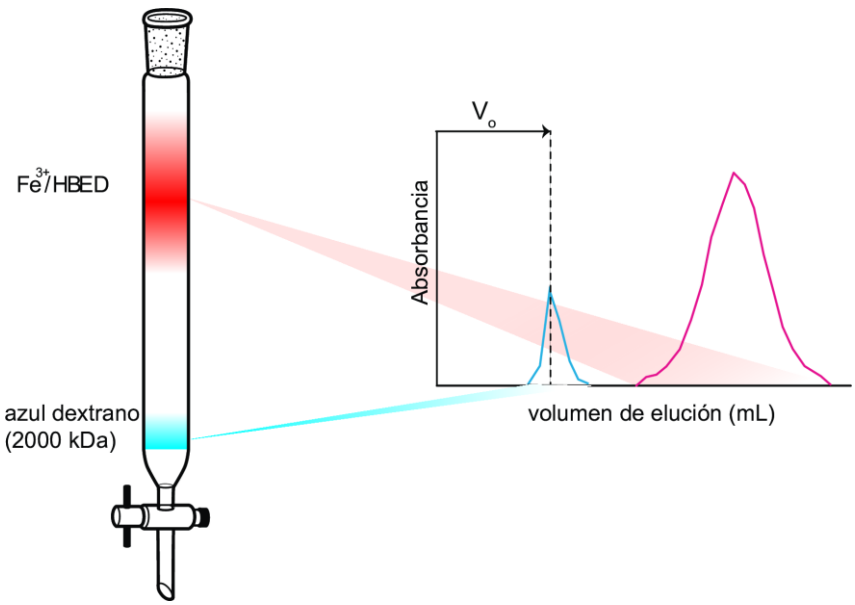

Figura I.8. Representación esquemática de la Cromatografía de filtración en gel empleada en esta Tesis. Fuente:Propia.

1.5 Métodos analíticos para la caracterización de agentes complejantes y complejos

Actualmente, la máxima capacidad complejante (MCC) del ligando, así como el metal soluble y complejado de los complejos son tres parámetros críticos que deben ser analizados previo a su aplicación en los cultivos.

1.5.1 Determinación de la máxima capacidad complejante de los ligandos

La MCC es un índice adecuado que se ha utilizado para discriminar ligandos con mayor capacidad para complejar micronutrientes en condiciones de suelo calizo. Su determinación se basa en el método descrito por Villén *y col*. (2007), evaluando la concentración del metal que queda en disolución tras la adición de volúmenes crecientes de una disolución de metal de concentración conocida a una disolución de ligando a pH 9. Previamente al análisis del metal por AAS (método 9.4, EU, 2003), se elimina el precipitado formado mediante filtración y se mineraliza el sobrenadante para la eliminación de la materia orgánica (método 9.3, EU, 2003). De esta forma lo que se obtiene es el punto de máxima complejación a partir de la intersección entre el segmento de complejación y el segmento de precipitación/coagulación como consecuencia del metal añadido (Figura I.9).

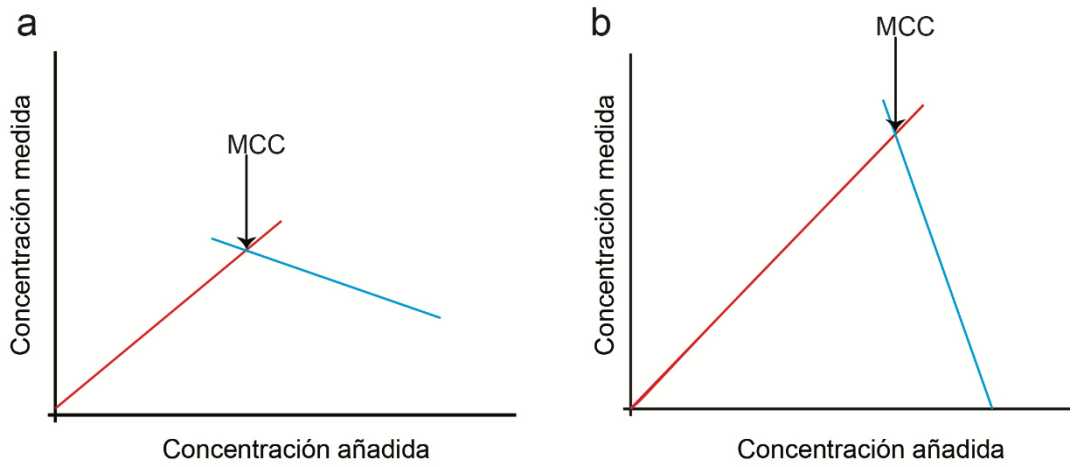

Figura I.9. Tipos de curvas de titulación de la máxima capacidad para agentes complejantes a pH 9. Fuente: Propia.

1.5.2 Determinación del elemento soluble

El contenido de elemento soluble es un requisito que debe ser analizado en los complejos que son utilizados como fertilizantes, el cual debe ser mayor al 5% para complejos sólidos y mayor al 2% para complejos líquidos de acuerdo a la legislación europea para fertilizantes (EU, 2003). Este parámetro es analizado por extracción en

agua, incluyendo la filtración de la solución a 0.45 µm (método 9.2, EU, 2003), seguido por la eliminación de la materia orgánica con H_2O_2 en medio ácido (método 9.3, EU, 2003) y posterior análisis por AAS (método 9.4). No obstante, las impurezas que contengan los complejos pueden influir en la solubilización del elemento, las cuales pueden no estar unidas al ligando. Este hecho ha sido previamente demostrado para quelatos (López-Rayo *y col.*, 2014).

Por ello, el análisis del elemento complejado (sección 1.5.3) debe ser analizado junto al del elemento soluble.

1.5.3 Determinación del elemento complejado

El contenido de metal complejado es analizado por el método EN 15962 (CEN, 2011), el cual consiste en la cuantificación del compuesto soluble (metal-ligando) a pH 9, previa eliminación del precipitado por filtración de la solución a 0.45 µm y de la materia orgánica como en el elemento soluble. De acuerdo con la legislación oficial europea (EU, 2003) el porcentaje de metal complejado debe ser mayor al 80% para los complejos usados como fertilizantes.

Los resultados del elemento soluble y complejado también permiten obtener la fracción de metal complejado (expresada como porcentaje de metal complejado con respecto al metal soluble) utilizada como un índice de la estabilidad del complejo (López-Rayo *y col.*, 2014). Sin embargo, la efectividad de los complejos no solo depende de la fracción complejada sino también de otros factores como: pH, el metal, tipo de ligando, pureza del agente complejante, peso molecular, grupos funcionales y relación metal: ligando.

1.6 Parámetros físico-químicos que influyen en la efectividad de los complejos

En distintos estudios se sugiere que los complejos de baja estabilidad son recomendados en hidroponía, mientras que los complejos más estables son más eficaces en suelos calizos (Carrasco *y col.*, 2012; López-Rayo *y col.*, 2015). Sin embargo, al igual que en el caso de los quelatos, es de suma importancia evaluar los siguientes parámetros para un mejor entendimiento en la preparación y efectividad de los complejos cuando son aplicados a los cultivos:

1) el pH del medio; ya que cuando se aplican complejos de micronutrientes en disolución para aplicación foliar, hidroponía o fertirrigación su pH se encuentra habitualmente entre 4 y 6, pero cuando son aplicados en un suelo calizo el pH puede incrementar a 8.5, influyendo en su estabilidad. En estudios previos, los LS y el G6 han presentado una alta capacidad para mantener Mn, Fe o Zn solubles en soluciones

alcalinas (Lucena *y col*., 2010; Benedicto *y col*., 2011; López-Rayo *y col*., 2014), contrario a la estabilidad variable que han mostrado en condiciones de suelo calizo. Mientras que su baja estabilidad ha sido demostrada por diferentes autores (Goos y Germain, 2001; López-Rayo *y col*., 2015), otros han confirmado su capacidad para proveer micronutrientes en las mismas condiciones (Cieschi *y col*., 2016; Martín-Ortiz *y col*., 2016; Martín-Fernández *y col*., 2017a). Por ello, Lucena *y col*. (2010) indicaron que los complejos deben ser estables en disolución durante, al menos varios días, manteniendo el metal soluble en el rango de pH que son aplicados.

2) El tipo de metal; así ligandos como los LS y G6 han mostrado una baja estabilidad para mantener Zn en solución a un pH alto, mientras que con Mn han resultado más estables que el EDTA y HEEDTA (López-Rayo *y col*., 2012). La precipitación de formas insolubles de Zn de esos complejos es causada por la menor estabilidad a un pH mayor a 8. La formación de complejos fuertes o débiles con lignosulfonatos y leonardita depende del estado de oxidación del Fe, de acuerdo con Carrasco *y col.* (2012) y Kovács *y col.* (2013), los complejos más fuertes son los formados a partir del Fe^{3+}

3) El tipo de ligando y la pureza del complejante; también son parámetros importantes en la efectividad de los complejos. Según López-Rayo *y col*. (2014) un complejo comercial de Mn:G6 presentó menor estabilidad en disolución alcalina en comparación con un complejo comercial de Mn:G7 que contenía impurezas de G6, indicando que el G7 puro sería mejor ligando que el G6. Rodríguez-Lucena *y col*. (2009) también demostraron que la efectividad depende del tipo de ligando, ya que los LS de eucalipto fueron más eficientes que los de abeto para proveer Fe a plantas de tomate y pepino fertilizadas por aplicación foliar.

4) El peso molecular de los complejos; también influye en su efectividad, ya que se ha observado que complejos de Zn:LS de bajo peso molecular presentan mejor capacidad que complejos de Zn:LS de alto peso molecular para proveer Zn en plantas de judía (Benedicto *y col*., 2011; Minnocci *y col*., 2018) fertilizadas por aplicación foliar, así como en plantas crecidas en condiciones de suelo calizo (Cieschi *y col*., 2016).

5) Los grupos funcionales; fenoles, carboxilos, hidroxilos y azufre (S) orgánico intervienen en mayor o menor proporción en la capacidad para complejar y proveer micronutrientes a las plantas. Benedicto *y col*. (2011) demostraron que un LS con mayor contenido de fenoles, presentó una mayor capacidad para complejar Zn, así como para proveer este micronutirentes a las plantas por medio de aplicación foliar. Martín-Ortiz *y col*. (2009) también determinaron que un LS con un mayor contenido de

grupo fenólicos y azufre presentó una mayor capacidad para proveer Zn bajo condiciones de hidroponía a plantas de maíz y trigo. Igualmente Rodríguez-Lucena *y col*. (2011) demostraron la mayor capacidad para proveer Fe de un compplejo con LS con un mayor contenido de grupos sulfónicos y carboxílicos mediante aplicación foliar en plantas de pepino. Sin embargo, también sugirieron que eran menos estables que un LS con un mayor contenido de grupos hidroxilo. Pang *y col*. (2008) también observaron una mayor capacidad de complejación en LS de pino modificados por hidroximetilación debido al incremento en el contenido de grupos hidroxilo.

6) La influencia de la relación metal:ligando y formación de especies poliméricas; en la estabilidad de los complejos también ha sido confirmada para diferentes ligandos, ya que dependiendo de esta relación se pueden formar amplia variedad de especies químicas. Por ejemplo, Silva *y col*. (2009) confirmaron que una alta relación molar de Fe^{3+}:citrato a pH neutro promovió la formación de complejos oligoméricos, sin embargo una baja relación molar a pH 9 originó la formación de complejos mononucleares de Fe. Estudios previos (Garcia-Mina, 2006; Stevenson, 1982) también demostraron que complejos con altas relaciones Fe:ácidos húmicos presentaban menos estabilidad que cuando las relaciones fueron más bajas, debido al menor contenido de grupos funcionales libres. Un resultado similar fue descrito por Rodríguez-Lucena *y col*. (2010) para complejos de Fe:humatos y Fe:LS, los cuales pueden presentar diferente estabilidad dependiendo de los sitios de unión de estos polímeros y de que los metales se unen primero a los sitios que proporcionan una mayor estabilidad. Esta hipótesis fue corroborada por Carrasco *y col*. (2012) a partir del análisis de diferentes relaciones Fe:LS por Mössbauer. Varios estudios (Spiro *y col*., 1967; Tonković *y col*., 1983) también demostraron que los azúcares y el citrato con Fe^{3+} forman complejos de alta estabilidad, debido a la formación de especies poliméricas que previenen la precipitación del Fe.

II. OBJETIVOS

La presente Tesis Doctoral pretende contribuir a mejorar el conocimiento científico sobre la relación entre las características fisicoquímicas de los complejos y su habilidad para proveer micronutrientes a las plantas bajo diferentes condiciones agronómicas. Existen varios estudios que han evaluado la efectividad de los complejos dependiendo de su origen o composición, pero pocos han analizado la relación con las características fisicoquímicas del agente complejante y los cambios sufridos después del proceso de complejación. La resonancia magnética nuclear y la espectroscopía de infrarrojos por transformada de Fourier son técnicas que han permitido elucidar los cambios estructurales ocurridos después de la complejación. Asimismo, la cromatografía de filtración en gel (GFC) y cromatografía de exclusión por tamaño de alta resolución (HPSEC) han permitido distinguir LS, quelatos de Fe y fracciones de Fe-humatos que pueden ser usados como fertilizantes agronómicos. Sin embargo, GFC y HPSEC no han sido utilizadas hasta ahora para la evaluación de complejos metálicos a partir de lignosulfonatos, heptagluconato y gluconato, siendo estos complejantes de interés agrícola debido a su alta biodegradabilidad y bajo costo. Además, los estudios de aplicaciones de G6 y G7 sobre plantas son limitados o contradictorios, ya que existen varios parámetros que pueden influir en la efectividad de los complejos como es el caso de la relación metal: ligando. Este parámetro puede influir en la estabilidad de los complejos y en la formación de especies mononucleares o polinucleares, como ha sido previamente demostrado para otros agentes complejantes como LS, humatos y citrato.

Por todo ello, se ha planteado el siguiente objetivo general:

Identificar los parámetros físico-químicos que influyen en la efectividad de agentes complejantes, tales como los lignosulfonatos, heptagluconato y gluconato para su uso como fertilizantes de Fe, Mn y Zn, mediante el empleo de técnicas de caracterización química y ensayos biológicos.

Para lograr este objetivo general se han planteado los siguientes objetivos específicos:

1.- Obtener una caracterización general y la distribución de peso molecular por GFC y HPSEC de lignosulfonatos (LS) obtenidos a partir de diferentes especies de madera y procesos al sulfito complejados con Fe^{3+}/Fe^{2+}, Mn^{2+} y Zn^{2+}. De este modo, se podrán identificar los factores involucrados en la efectividad de LS, y por tanto aquellos con mayor capacidad fertilizante.

2.- Evaluar la eficacia de complejos de Fe de heptagluconato (G7) y gluconato (G6) aplicados en diferentes relaciones Fe:ligando en plantas de estrategia I, para proveer este micronutriente en condiciones de hidroponía y de suelo calizo.

3.- Evaluar los parámetros que afectan a la estabilidad de complejos de Mn a partir de su máxima capacidad complejante, fracción complejada, caracterización estructural y distribución del peso molecular para evaluar la eficacia de heptagluconato y gluconato como fertilizantes de dicho micronutriente.

4.-Comprobar la influencia de la relación Mn:ligando en la nutrición de plantas deficientes de Mn bajo condiciones de hidroponía y suelo calizo fertilizadas con heptagluconato y gluconato.

Objectives

This doctoral thesis is a contribution to the enhancement of the scientific knowledge about the relationship between the physicochemical characteristics of the complexes and their ability to provide micronutrients to plants under different agronomic conditions. Previous studies have evaluated the effectiveness of the complexes based on their nature or their chemical composition, but only few studies have analyzed the relation between the physicochemical characteristics of the complexing agents and the changes suffered after the complexation process. These structural changes have been elucidated by spectroscopical techniques such as nuclear magnetic resonance and Fourier transform infrared. The gel filtration chromatography (GFC) and the high-performance size exclusion chromatography (HPSEC) techniques have also allowed identifying lignosulfonates, chelates and Fe-humates fractions for their application as fertilizers. However, the evaluation of micronutrient complexes from lignosulfonate (LS), heptagluconate (G7) and gluconate (G6) have not been evaluated by these techniques, whose ligands are of high agricultural interest for their low-cost and low environmental impact. On the other hand, studies of G6 and G7 applied to plants have been limited or not conclusive, since parameters such as the metal:ligand ratio assayed can influence the complex effectiveness. This parameter can influence the complex stability, and the mononuclear and polynuclear complexes formation, as previously demonstrated for other complexing agents such as LS, humates, and citrate.

For all these reasons, the general objective of this thesis was:

To identify the physicochemical parameters that influence the effectiveness of complexing agents such as lignosulfonates, heptagluconate and gluconate to be used as Fe, Mn, and Zn fertilizers, using chemical characterization tools and biological assays.

To achieve the general objective, the following specific objectives was set:

1.- To obtain an overall characterization and the molecular weight distribution by GFC and HPSE of lignosulfonates (LS) obtained from different wood sources and sulfite pulping processes complexed with Fe^{3+}/Fe^{2+}, Mn^{2+} y Zn^{2+}. Thereby, the factors involved in the effectiveness of LS can be elucidated, and consequently, those with potential fertilizer capacity identified.

2.- To evaluate the efficacy of different Fe complexes of heptagluconate (G7) and gluconate (G6) at several Fe:ligand molar ratios, applied as Fe fertilizers in strategy I plants grown under hydroponics and calcareous soil conditions.

3.- To evaluate the parameters that affect the stability of the Mn complexes by means of their maximum complexing capacity, complexed fraction, structural characterization and molecular weight distribution, to evaluate the efficacy of heptagluconate and gluconate as Mn fertilizers.

4.-To evaluate the influence of the Mn:ligand ratio in the nutrition of Mn-deficient plants grown under hydroponics and calcareous soil conditions and fertilized with the heptagluconate and gluconate Mn complexes.

III. ASSESSING METAL–LIGNOSULFONATES AS FERTILIZERS USING GEL FILTRATION CHROMATOGRAPHY AND HIGH-PERFORMANCE SIZE EXCLUSION CHROMATOGRAPHY

Resumen

Los lignosulfonates (LSs) son subproductos de la industria del papel usados como fertilizantes biodegradables. Sin embargo, la capacidad de los metal-LSs para proveer micronutrientes se relaciona con la estabilidad del complejo y la proporción del metal complejado. En este trabajo se evaluaron estos parámetros usando técnicas espectroscopicas de ultravioleta-visible (UV-vis), infrarroja con transformada de Fourier (FTIR) y resonancia magnética nuclear de carbono (^{13}C NMR), junto con la cromatografía de filtración en gel (GFC) y cromatografía de exclusion por tamaño de alta resolución (HPSEC), para diferentes LSs de abeto, eucalipto y pino. Las técnicas de GFC y HPSEC señalaron que la cantidad y tipo de metal complejado en los LSs depende del peso molecular, pH y el proceso de producción de celulosa al sulfito. Ambas técnicas indicaron que un LS de bajo peso molecular enriquecido con grupos fenólicos presenta la más elevada capacidad complejante. También, se evidenció la formación de compuestos de alto peso molecular en los Fe(III)/LSs, mientras los complejos de Zn(II)/LS y Mn(II)/LS no formaron agregados. El fraccionamiento de metal-LSs proporcionó información importante para identificar LSs con mayor capacidad fertilizante y para evaluar la efectividad de sus complejos.

Abstract

Lignosulfonates (LSs) are by-products from the paper industry used as biodegradable fertilizers. However, metal–LSs ability to provide micronutrients to crops is related to the stability of the complex and the amount of metal complexed. This work evaluated these parameters using ultraviolet–visible (UV–Vis), Fourier-transform infrared (FTIR), and ^{13}C-nuclear magnetic resonance (NMR), along with gel filtration chromatography (GFC) and high-performance size exclusion chromatography (HPSEC), for different spruce, eucalyptus, and pine LSs. GFC and HPSEC pointed out that the amount and type of complexed metal in the LS depends on the molecular weight, pH, and sulphite pulping processes. Both techniques indicated that the low molecular weight LS enriched with phenolic groups has the highest Fe(III) complexing capacity. Also, Fe(III)/LSs showed the formation of high molecular weight compounds, whereas Zn(II)/LS and Mn(II)/LS complexes did not form aggregates. Metal–LS fractionation provided considerable information to identify LSs with potential fertilizer capacity and to assess the effectiveness of their complexes.

3.1. Introduction

Iron (Fe), manganese (Mn), and zinc (Zn) are essential micronutrients for many biological processes, which affect crop yield and food quality (Marschner, 2012). However, the high pH and bicarbonate buffer in calcareous soils often limits their bioavailability (Lucena, 2000). The application of synthetic chelates is the most efficient practice to treat micronutrient deficiencies (Lucena *et al.*, 2010), but they are expensive, recalcitrant and may mobilize heavy metals. So, the search for biodegradable, low-cost and environmental-friendly alternatives, such as the lignosulfonates (LSs) is needed (Collins *et al.*, 2019).

LSs are by-products from the paper industry, obtained from softwood (pine and spruce wood) or hardwood (eucalyptus wood) (Lin and Dence, 1992), which are extracted by the sulphite pulping process using excess aqueous solution of bisulphite and one hydroxide (Collins *et al.*, 2019). These products were included in the list of authorized ligands for micronutrient fertilization in the Directive 223/2012 for fertilizers updated from the Fertilizer Directive 2003/2003 (EU, 2003). The LS complexes maintain micronutrient availability in the soil better than inorganic salts (Martín-Ortiz *et al.*, 2016) due to the presence of a wide variety of functional groups, such as alcoholic hydroxyl, carboxylic, phenolic, and sulfonic groups that are present in their structure (Pang *et al.*, 2008; Rodríguez-Lucena *et al.*, 2011). The content of these functional groups in the LS depends on the plant species isolated (softwood and hardwood) and the sulphite pulping processes used, such as phenolation, sulfonation, or oxidation, which increase the amount of phenolic, sulfonic, and carboxylic groups, respectively (Benedicto *et al.*, 2011; Collins *et al.*, 2019), as well as ultrafiltration. Consequently, these processes can modify the molecular weight (MW) of the LS. For instance, when the ultrafiltration method applies to the LS, its MW is considerably low, improving the complexing capacity and ability to provide Zn to plants (Benedicto *et al.*, 2011). In contrast, the LS with a high MW presents better Fe-chelating ability (Rodríguez-Lucena *et al.*, 2011; Wang, Xue and Liu, 2012). Thus, fractionation by ultrafiltration can enhance the MW of the LS, but it is quite costly and difficult to implement on an industrial scale (Collins *et al.*, 2019).

Several researchers have evaluated the effectiveness of metal–LS complexes depending on their origin or their composition (González *et al.*, 2015; Martín-Ortiz *et al.*, 2016; Minnocci *et al.*, 2018), but only a few studies have investigated the relationship between the physicochemical properties of the LS and its capacity to provide nutrients to plants under different growth conditions. An increase in the complexing capacity of

the LSs have been demonstrated due to the phenolic (Benedicto *et al.*, 2011), hydroxyl (Pang *et al.*, 2008), sulfonic (Martín-Ortiz *et al.*, 2009; Rodríguez-Lucena *et al.*, 2011), and carboxylic (Gonçalves and Benar, 2001; Rodríguez-Lucena *et al.*, 2011) groups present in their structure. Regarding the wood species origin of the LS, the eucalyptus LS showed better ability to provide Fe(III) and Zn(II) to the plants than the spruce LS (Benedicto *et al.*, 2011; Rodríguez-Lucena *et al.*, 2011; Cieschi *et al.*, 2016), while Martín-Ortiz *et al.*, (2009) obtained better results for Zn(II) with the spruce LS than the eucalyptus LS. Therefore, the LS complexing capacity depends on the structural changes that occur during the sulphite pulping processes (Benedicto *et al.*, 2011) and the plant species (Myrvold, 2016), affecting the molecular weight distribution (MWD). In this work, we use an extensive study to highlight the physicochemical properties of LS that influence its complexing capacity, using LS extracted from different sulphite pulping processes and plant species, such as pine LS, which has not been previously considered.

Nowadays, quantification of the micronutrients complexed by the LS is a critical point of its characterization before LS application to crops (Villén *et al.*, 2007). Furthermore, the combination of several spectroscopy techniques, including Fourier-transform infrared spectroscopy (FTIR), ^{13}C nuclear magnetic resonance (NMR), and ultraviolet–visible (UV–vis) spectroscopy (Rodríguez-Lucena *et al.*, 2011), are also useful tools for elucidating the structural changes that occur in the LS after the complexation process with metals.

Size exclusion chromatography (SEC) is a technique for polymer separation according to MW. SEC includes gel filtration chromatography (GFC), which has been applied to determine the MWD of LSs on Sephadex since the early 1960s, and high-performance size exclusion chromatography (HPSEC), which has better chromatographic resolution and shorter analysis time (Lin and Dence, 1992). Studies with HPSEC have shown that the MW and fractions from softwood LSs (spruce) are higher than hardwood LSs from eucalyptus (Fredheim *et al.*, 2002; Braaten *et al.*, 2003). Also, the analysis of LSs using a combination of ion-pair chromatography and SEC has been applied to distinguish LSs that can be used in agronomical fertilizers (Brudin *et al.*, 2008). Meanwhile, GFC on Sephadex has not only been applied to determine the MWD of LSs but also the analysis of fertilizers, such as Fe chelates and Fe–humic complexes (Boxema, 1979; Pérez-Sanz *et al.*, 2006). According to our knowledge, these techniques have not been used for the evaluation of metal–LS complexes. Thus, the novelty of this study not only consisted of elucidating the physicochemical properties of the LS and their complexes using GFC and HPSEC, but also the analysis of their stability at different pH values,

which can influence the ionization of the functional groups of the LS and, consequently, the complexed metal.

This study aimed to investigate the MW fractioning of several LSs obtained from different wood sources and sulphite pulping processes complexed with Fe(II)/(III), Mn(II), and Zn(II). For this purpose, the stability of the metal–LS complexes and the final amount of the metal complexed in each fraction were evaluated using two SEC methods at different pH values. This knowledge will contribute to a better understanding of the chemical reactions affecting LS complexation with micronutrients.

3.2. Materials and methods

Five LSs obtained from the sulphite pulping process of softwood (maritime pine, *Pinus pinaster L.*), provided by Tembec Innovation (France), were analysed. Three were ammonium salts (P1, P2, and P3), one was a magnesium salt (P4), and the fifth was a potassium salt (P5). Moreover, a hardwood (*Eucalyptus globulus L.*) calcium LS (E1) and a softwood (spruce, *Picea abies* L.) calcium LS (S1) were provided by Lignotech Iberica S.A. (Torrelavega, Cantabria, Spain). All reagents used to form the complexes [$FeCl_3 \cdot 6H_2O$ (PA, Merck, 99%), $FeSO_4 \cdot 7H_2O$ (PA, Merck, 99%), $ZnSO_4 \cdot H_2O$ (PA, Panreac, 99%) and $MnSO_4 \cdot H_2O$ (PA, Panreac, 98%)] were reagent grade, and the water used was grade I, free of organic contaminants.

3.2.1. Complexing capacity of lignosulfonates and preparation of the complexes

The maximum complexing capacity (MCC) of the LS with Fe (II)/Fe(III), Mn(II), and Zn(II) was evaluated by determining the amount of the element that remained in the solution at pH 9 after 1 day in the dark (Villén *et al.*, 2007). Next, the complexes were prepared based on 90% of their MCC, which was previously determined, and then lyophilized. The soluble element in the lyophilized samples was determined following method 9.2 (EU, 2003) by extraction in water, including use of a 0.45-µm filter for filtration of the solution, followed by removal of the organic compounds with H_2O_2 under acidic conditions and then measurement by atomic absorption spectroscopy (AAS). The complexed metal in the solid samples was analysed by the method EN 15962 (CEN, 2011), which describes a similar sample preparation method as the soluble element method, but it involves increasing the pH to greater than 9 prior to using the 0.45-µm filter for filtration of the solutions.

3.2.2. Physicochemical characterization of pine lignosulfonates

The dry weight (DW) content was evaluated by drying 2 g of the product in a stove at 60ºC until constant weight. The pH of the LS was provided by Tembec Innovation. The phenolic groups, organic S, and lignosulfonic acid (LSA) content of the lyophilized LS were determined according to EN 16109 (CEN, 2012) by the difference in UV–vis absorbance, oxidation of inorganic sulphur to sulphate, and UV–vis absorption at 232.5 nm, respectively. The carboxylic groups were analysed from an aqueous, potentiometric titration (Gosselink *et al.*, 2004), and the elemental analysis of nitrogen (N), carbon (C), hydrogen (H), and sulphur (S) was performed using a LECO CHNS-932 analyser (Leco Corporation, USA). The FTIR spectra of the lyophilized LS and its complexes were obtained by the KBr pellet method on a Bruker IFS66v spectrometer (Germany). The ^{13}C-NMR spectra of the LS and the Zn (II)/LS complexes dissolved in deuterated water were recorded using a Bruker DRX 500 MHz (Germany) with power gate decoupling sequence (zgpg30). The number average molecular weight (av M_n) and the average molecular weight (av MW) of the LS were determined by HPSEC, as described in section 2.4, using the following equations:

$$av\ M_n = \sum N_i M_i / \sum N_i \quad (1)$$

$$av\ Mw = \sum N_i {M_i}^2 / \sum N_i M_i \quad (2)$$

where N_i and M_i are the number of chains and the molecular weight of the chains, respectively (Agilent Technologies, 2015).

3.2.3. Gel filtration chromatography of pine lignosulfonates

The LS and its complexes were fractionated using a glass column (1.0 x 30 cm) packed with Sephadex® G-100 (molecular weight cut-off~1–100 kDa, 40–120 µm particle size, Sigma Aldrich). The exclusion volume (V_o) was determined using blue dextran 2000 (MW~2000 kDa), and the total permeation volume (V_p) was determined with iron(III) N,N'-bis(2-hydroxybenzyl)ethylenediamine-N,N'-diacetate (HBED/Fe(III), MW~440 Da). For that purpose, a mixture of both compounds was prepared (0.2%,

w/v), and 125 µL were added to the column for elution. The eluents used were NaCl solutions at different concentrations ranging from 0.1 M to 2.0 M at pH 6, which was used to avoid changes in the pH of the synthesized complexes (Fig. S1, Supplementary data I). Finally, the lyophilized LS and its complexes were dissolved in 0.1 M NaCl (0.6%, w/v) at pH 6 prior to the filtration (0.22-µm filter) of the solutions (to remove solids and to avoid the risk of blockage). Then, an aliquot of 125 µL was loaded onto the column and eluted by gravity-flow at room temperature. The fractions were recorded using a Spectrostar nano microplate reader (BMG Labtech, Ortenberg, Germany) at 280 nm (characteristic absorption corresponding to the LS), and the metal concentration of each fraction was measured by AAS.

3.2.4. High-performance size exclusion chromatography of different wood lignosulfonates

The HPSEC fractionation of the LSs E1, S1 and P2 and their complexes was performed by modification of the methodology described by Brudin *et al*. (2008). Chromatograms were recorded at 280 nm in a Waters 2695 Alliance high-performance liquid chromatography system equipped with a Waters 996 photodiode array with a Biosep Sec-S2000 column (300–1 kDa, 300 x 7.80 mm, 5-µm particle size, Phenomenex). The complexes were prepared based on 90% of the MCC from the LS (0.17%, w/v) and the pH was adjusted to 6.0 and 9.0, followed by filtration (using a 0.22-µm filter) of the solutions. The injection volume was 20 µL. The mobile phase composition was 0.1 M lithium chloride in 90 mM tetrabutylammonium hydroxide (TBAOH, 40 wt% in water) at pH 6.0, with a flow rate of 1 mL min^{-1} at 30°C. The exclusion time (T_o) was determined with blue dextran 2000 (MW~2000 kDa), and the total permeation time (T_p) was determined with HBED/Fe(III) (MW~440 Da). Two standard LSs (Sigma-Aldrich, St. Louis, MO, USA), designated as E2 (av MW~18000 Da) and S2 (av MW~52000 Da), were also included for the calibration.

3.3. Results and discussion

3.3.1. Physicochemical characterization of the lignosulfonates

P4 contained a higher amount of phenolic groups than the rest of the pine LSs (Table III.1), but lower than the E1 and the S1, which were previously characterized (Benedicto *et al.*, 2011; Rodríguez-Lucena *et al.*, 2011). This result was corroborated by the FTIR spectrum (Fig. III. 1a), with the strongest bands associated with aromatic (1619, 1513, and 1403 cm^{-1}) and hydroxyl (3400 cm^{-1}) vibrations, as well as its ^{13}C NMR spectrum (Fig. III.S2a, Supplementary data I), with most abundant peaks at 160–100 ppm (aromatic carbons). Moreover, P4 presented the lowest LSA content and MWD than the rest of the pine LSs (Table III.1). According to Benedicto *et al.* (2011), all of these characteristics can improve its complexing capacity. The lowest MW seems to be the main property that contributes to the high phenolic group content in P4, as has already been pointed out in the fractionation of lignin (Collins *et al.*, 2019). The highest amount of carboxylic groups of P1 was supported by the bands assigned to carboxylic groups at 1777–1701 cm^{-1} in its FTIR spectrum (Fig. III.1a) and to the peaks at 181–164 ppm (carboxyl carbons) in its ^{13}C NMR spectrum (Fig. III.S2a, Supplementary data I). The lowest amount of organic S was found with P2, while P5 and P3 had the highest among the pine LSs, but not higher than E1 and S1. Also, P3 showed the highest LSA content than the rest of the pine LSs and E1, but lower than S1. Thus, these parameters of P3 and P5 can also improve their complexing capacity, as previously demonstrated by S1 and E1 (Martín-Ortiz *et al.*, 2009; Rodríguez-Lucena *et al.*, 2011). The low content of carboxylic and phenolic groups presented by P5 was supported by the weak bands assigned to hydroxyl and aromatic groups in its FTIR spectrum (Fig. III.1a) and the absence of aromatic carbon peaks in its ^{13}C NMR spectrum (Fig. III.S2a, Supplementary data I). Besides, elemental analysis of the pine LS indicated the following composition range (%): C, 38–44%; H, 4–6%; and S, 5–8%, which is in good agreement with other LSs that were previously characterized (Ahvazi *et al.*, 2016). The high S percentage in the LS is expected from the sulphite pulping process applied. The ammonium sulphite pulping applied to P1, P2, and P3 increased their N content (4–5%) compared to P4 (0.2%) and, P5 (1.2%).

Table III.1. Physicochemical characteristics of the lignosulfonates. Values are given as means ± standard error (*n*=3).

Parameters	E1*	S1*	P1	P2	P3	P4	P5
pH†	4.30	3.60	3.72	4.00	3.14	3.22	7.88
DW (g Kg^{-1} LS)	537†	503†	495 ± 1.3	496 ± 1.3	506 ± 1.9	521 ± 0.3	458 ± 0.2
Organic-S (g Kg^{-1} LS DW)	51.0†	55.0†	30.3 ± 2.3	25.4 ± 1.7	36.8 ± 1.9	32.5 ± 0.8	34.4 ± 0.2
Phenolic (g Kg^{-1} LS DW)	19.0†	19.0†	11.8 ± 0.3	10.4 ± 0.6	10.7 ± 0.8	14.7 ± 0.2	10.1 ± 0.3
Carboxyl (g Kg^{-1} LS DW)	35.0†	26.0†	50.0 ± 0.9	40.6 ± 0.3	42.2 ± 1.6	37.5± 0.1	36.1 ± 2.8
LSA content (g Kg^{-1} LS DW)	587± 0.3	835 ± 11	638 ± 21	612 ± 80	663 ± 90	461 ± 26	630 ± 6.6
Fe (mg Kg^{-1} LS DW)	24.8 ± 4.9	8.53 ± 5.6	15.0 ± 40	20.8 ± 9.3	5.80 ± 8.6	534 ± 11	123 ± 18
Mn (mg Kg^{-1} LS DW)	95.4 ± 5.0	31.0 ± 7.6	270 ± 8.9	133 ± 8.9	90.7 ± 13	649 ± 34	73.6 ± 2.0
Zn (mg Kg^{-1} LS DW)	15.3 ± 3.5	13.0 ± 6.6	bdl	bdl	bdl	bdl	bdl
av M_n (Da)	522	5698	5289	5285	5726	2488	6535
av MW (Da)	6275	25732	39658	39215	43005	17793	48947

*Benedicto et al. 2011; Rodríguez-Lucena et al. 2011. DW, dry weight. LSA, lignosulfonic acid content. av MW, the weight average molecular weight. av M_n, the number average molecular weight. †The standard deviation of the means does not show because of the data was provided by the company. bdl:below detection limit.

3.3.2. Complexing capacity of the lignosulfonates

The results previously presented in Table III.1 confirmed the differences in the content of organic S and the carboxylic and phenolic groups of the LS, which can influence its complexing capacity. Therefore, the MCC was also determined. The highest complexing capacity was found for the LS with Fe(III) compared to the divalent metals (Table III.2), especially P4, which is due to its high content of phenolic groups and low MW (Benedicto *et al.*, 2011). High complexing capacity was also obtained by Pang *et al*. (2008), who used pine LS modified from hydroxymethylation due to its increase in hydroxyl group content. Regarding the MCC of divalent metals, P5 was the best complexing agent for Zn(II) and Fe(II) compared to the rest of the pine LSs, which was attributed to the combined effect of both organic S content (Martín-Ortiz *et al.*, 2009) and high MWD (Rodríguez-Lucena *et al.*, 2011). However, the MCC of P5 was lower than S1 and E1, which was previously determined (Benedicto *et al.*, 2011; Rodríguez-Lucena *et al.*, 2011). This is due to the low amount of phenolic groups presented by P5 compared to S1 and E1 (Table III.1). For Mn, the highest complexing capacity of P3 was attributed to the high organic S (Martín-Ortiz *et al.*, 2009) content compared to the rest of the pine LSs, due to its higher carboxylic group content compared to S1 and E1 (Gonçalves and Benar, 2001; Benedicto *et al.*, 2011).

Table III.2. The maximum complexing capacity (MCC) of lignosulfonates with Fe (III), Fe (II), Zn (II) and Mn (II). Values are given as means ± standard error.

Products	Fe (III)	Fe (II)	Zn (II)	Mn (II)
		g Kg^{-1} LSA DW		
E1[*]	287 ± 2.0	180 ± 11	206 ± 2.9	137 ± 16
S1[*]	243 ± 9.0	178 ± 26	162 ± 4.3	168 ± 7.7
P1	180 ± 3.5	94 ± 2.4	84 ± 1.3	129 ± 3.6
P2	166 ± 5.0	115 ± 6.1	101 ± 3.8	147 ± 3.8
P3	186 ± 2.3	107 ± 3.2	106 ± 2.5	171 ± 1.4
P4	311 ± 5.1	98 ± 3.0	89 ± 3.6[†]	78 ± 0.8[†]
P5	183 ± 10	123 ± 5.0	110 ± 0.5	125 ± 2.1

[*]Benedicto et al. 2011; Rodríguez-Lucena et al. 2011. [†]Complexes discarded by precipitation during the preparation. The standard deviation of the means was calculated by the goodness of fit test with 4 to 9 degrees of freedom.

3.3.3. Structural characterization of the metal lignosulfonate complexes by **Fourier-transform infrared** *and ^{13}C nuclear magnetic resonance*

The FTIR spectra of the complexes were also analysed to confirm the structural changes after complexation of different metals. The Fe(III)/LS spectra presented bands at 3480, 1640, 1518, 1425, and 1109 cm^{-1} (Fig. III.1b), indicating interactions with the hydroxyl, carboxylic, aromatic, and sulfonic groups (Rodríguez-Lucena *et al.*, 2011; Pang *et al.*, 2016). The strong bands assigned to hydroxyl and aromatic groups for Fe(III)/P4 supported the highest complexing capacity (Table III.2), whereas Fe(III)/P5 presented an intense band at 623 cm^{-1}, associated with sulfonic groups.

The spectra obtained for Fe(II)/LS (Fig. III.1c), Zn(II)/LS (Fig. III.1d), and Mn(II)/LS complexes (Fig. III.1e) showed similar interactions as the Fe(III)/LS spectra, but with more intense bands for the sulfonic groups. The presence of sulfonic groups in the coordination of LSs with those metals have been previously observed in MnO_2 with lignin (Klapiszewski *et al.*, 2017) and sodium LS-stabilized ZnO nanoparticles (Pang *et al.*, 2016). Also, Zn(II)/P5 presented intense bands for the sulfonic groups, supporting its high Zn complexing capacity (Table III.2). The ^{13}C NMR spectra of Zn(II)/LS (Fig. III.S2b, Supplementary data I) indicated upfield shifts between 0.02 and 0.17 ppm for the aliphatic C–O (96 ppm) peak and downfield shifts between 0.01 and 1.97 ppm for the aliphatic COR (59 ppm) peak compared to the corresponding LS, confirming their interactions with Zn(II).

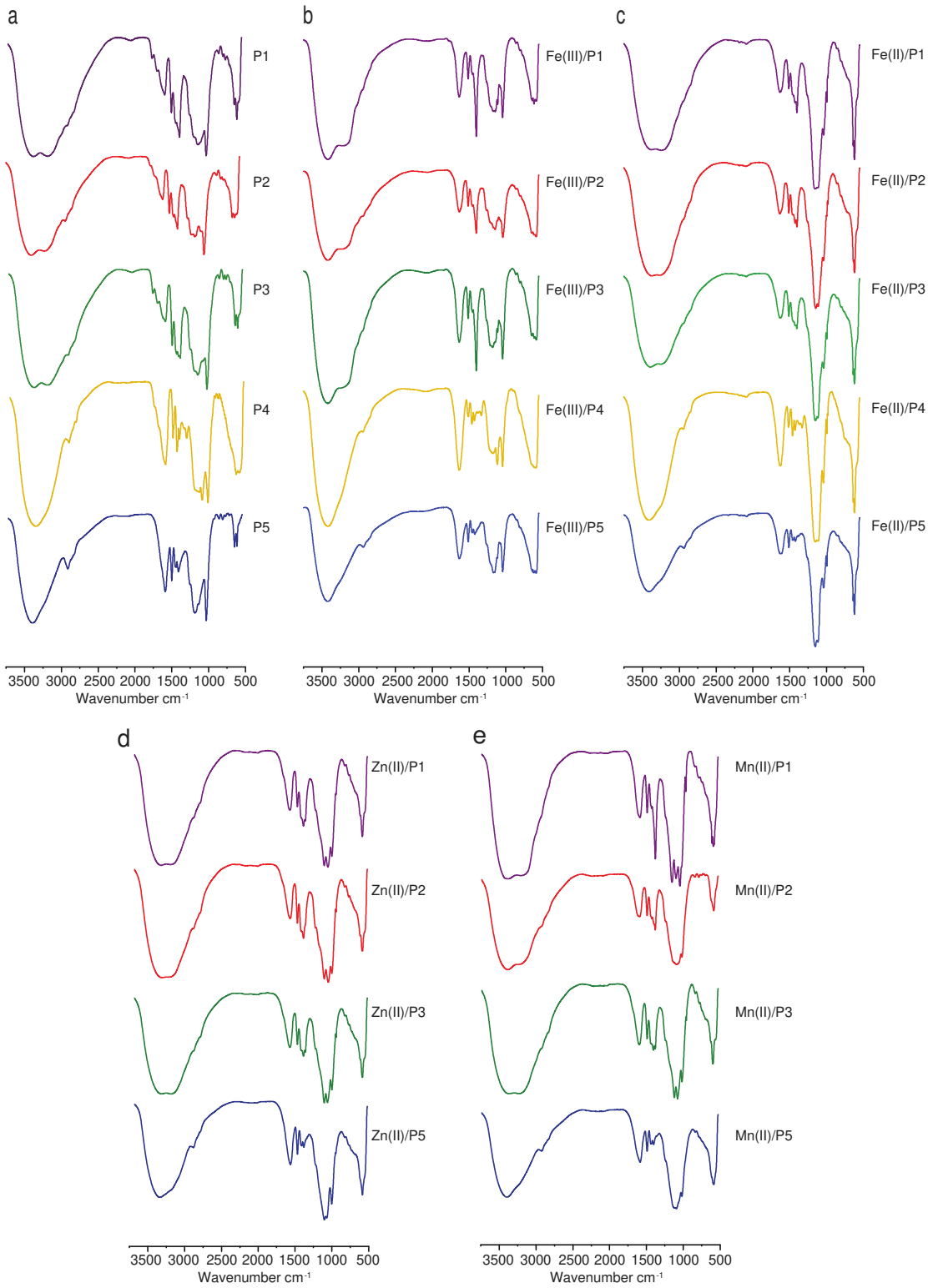

Fig. III.1. Fourier-transform infrared spectra of a) the pine LS and its complexes with b) Fe(III)/LS, c) Fe(II)/LS, d) Zn(II)/LS, and e) Mn(II)/LS.

3.3.4. Gel filtration chromatography of pine lignosulfonate

To obtain information about the MWD of pine LS obtained by different sulphite pulping processes and their complexes, the column of GFC was calibrated with dextran blue 2000 (2000 kDa) to indicate the limit of the high molecular weight fraction (HMW, >100 kDa) and low molecular weight fraction (LMW, <100 kDa), and with Fe(III)/HBED (440 Da), to determine the total volume (Fig. III.2a). The P1, P2, P3, and P5 chromatograms presented both HMW and LMW fractions (Fig. III.2b). In contrast, P4 presented only a LMW fraction, confirming that its MWD is lower than the rest of the pine LSs (Table III.1).

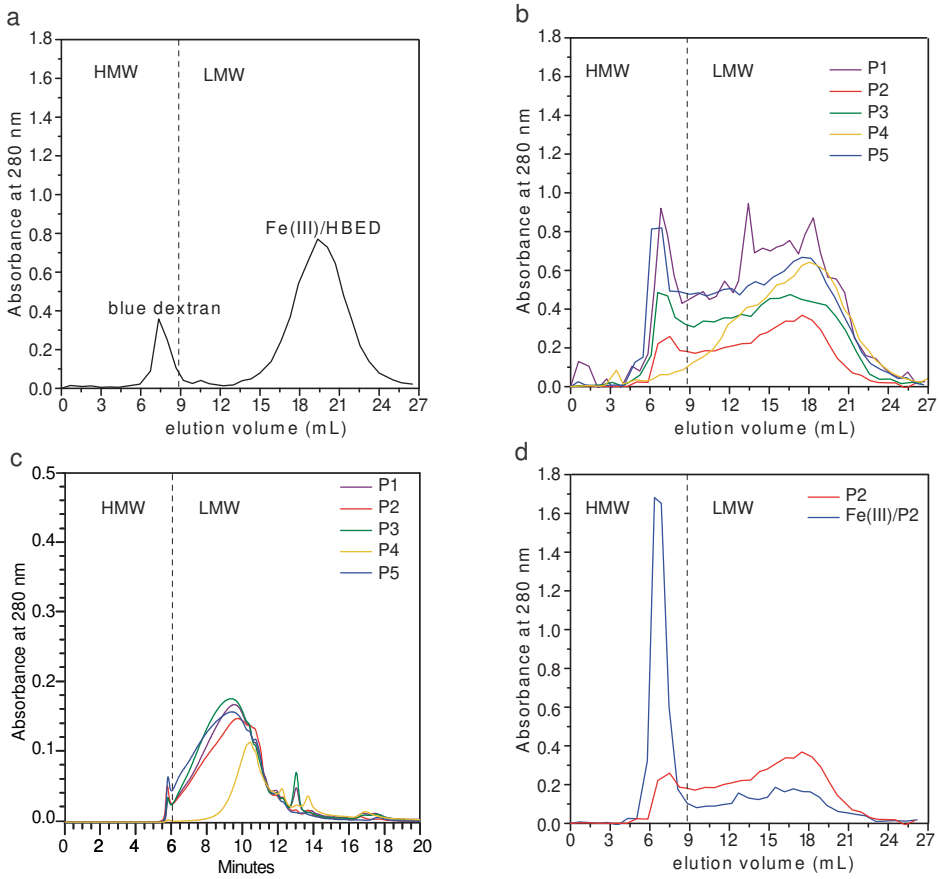

Fig. III.2. Chromatograms of elution profiles at pH 6. a) Calibration obtained by gel filtration chromatography. b) Comparison of lyophilized pine LS (0.6%, w/v) fractionated by gel filtration chromatography with 0.1 M NaOH and c) HPSEC with 0.1 M lithium chloride in 90 mM tetrabutylammonium hydroxide (TBAOH, 40 wt% in water). d) Fractionation of the Fe(III)/P2 complex compared to P2 LS by gel filtration chromatography. The exclusion time in gel filtration chromatography or elution time in HPSEC are indicated by dotted lines.

Chromatograms corresponding to the complexes of the LS were also analysed, indicating different binding of metals between both fractions. Thus, Fe(III)/LS showed a tendency to form HMW compounds (Fig. III.2d), possibly due to the formation of polynuclear iron-oxyhydroxide complexes surrounded by the LS structure, as previously showed by Fe–humic complexes (Kulikova *et al.*, 2017; Cieschi *et al.*, 2019), which are formed by the interaction of Fe(III) of the hydroxide with carboxylic and phenolic groups of the LS, according to Kulikova *et al.* (2017). The Fe(II)/LS chromatograms indicated a distribution between both HMW and LMW fractions. Although the Zn(II)/LS and Mn(II)/LS chromatograms showed various peaks, those revealed did not form HMW compounds.

To further analyses the stability of the complexes, the distribution of the metals in each fraction and the percentage of metal recovered were determined (Table III.3). Iron was found mainly in the HMW fractions for almost all Fe(III)/LS, confirming the tendency to form HMW compounds, as defined above. Also, the percentage of Fe recovered was higher than 71%, mainly for Fe(III)/P2, indicating that this complex could be an effective fertilizer applied to the leaf or under calcareous soil conditions because of its strong binding to the LS. Despite the highest complexing capacity of Fe(III)/P4 at pH 9, its percentage of Fe recovered at pH 6 was the lowest (25%) due to its low MWD and high phenolic group content (Table III.1). This can explain the low stability of E1 and, consequently, its effectiveness under hydroponic conditions, according to Carrasco *et al.* (2012). For Fe(II)/LS, the Fe was mainly distributed in the LMW fraction, except for Fe(II)/P5, which showed a uniform distribution between both fractions. It has been demonstrated that soft binding sites are most abundant in the LMW fraction, whereas hard binding sites could be found in the HMW fraction (Wu *et al.*, 2004). This suggests that Fe(II) is mainly bound to weak sites (sulfate) and to a lesser extent by hard sites such as carboxylic and phenolic groups (Tipping, 2002). This is consistent with the affinity of Fe(II) for both hard and soft sites (Smith *et al.*, 2002) and with the higher interaction of the Fe(II) complexes with sulfonic groups observed in the FTIR spectra (Fig. III.1c). The highest percentage of metal recovered by Fe(II)/P2 (94%), related to its higher complexed fraction (Table III.S1, Supplementary data I), also confirmed its high efficiency as a fertilizer.

Table III.3. Soluble metal contents (AAs) of pine lignosulfonates complexed with Fe (III), Fe (II), Mn (II) and Zn (II) obtained at the different elution fractions (high molecular weight (HMW) and low molecular weight (LMW) in gel chromatography; and metal recovery based on the total metal added.

Sample	% metal in HWM	% metal in LWM	% metal recovery
Fe (III)/P1	38.2	45.7	83.9
Fe (III)/P2	79.9	19.2	99.1
Fe(III)/P3	64.4	32.0	96.4
Fe(III)/P4	15.3	9.6	24.9
Fe(III)/P5	27.8	43.1	70.9
Fe(II)/P1	1.9	17.5	19.4
Fe(II)/P2	21.0	36.7	94.5
Fe(II)/P3	4.3	25.5	29.8
Fe(II)/P4	3.2	69.3	72.5
Fe(II)/P5	20.8	18.3	39.1
Zn(II)/P1	6.9	36.5	43.2
Zn(II)/P2	0.8	5.1	29.7
Zn(II)/P3	1.0	7.7	8.7
Zn(II)/P5	2.4	12.1	13.2
Mn(II)/P1	0.4	68.4	68.8
Mn(II)/P2	0.6	21.3	92.9
Mn(II)/P3	3.3	29.9	33.2
Mn(II)/P5	0	25.5	25.5

Zn(II) and Mn(II) were complexed mainly in the LMW fractions, as previously shown for Mn and Zn with colloidal organic matter (Worms *et al.*, 2010). This indicated the association of these metals with soft sites (Wu *et al.*, 2004), such as sulfonic groups (Smith *et al.*, 2002). These results are consistent with the FTIR spectra of Mn(II)/LS and Zn(II)/LS, with the strong bands at 1157–1109 cm^{-1} and 623 cm^{-1} (Fig. III.1) assigned to S–O stretching vibrations (Pang *et al.*, 2016; Klapiszewski *et al.*, 2017). Also, these complexes did not show the formation of HMW compounds due to their association with weak sites such as sulfonate, which are easily displaced (Wu *et al.*, 2004). Consequently, a low percentage of metal is recovered for almost all complexes, except to the Mn(II)/P2 and Mn(II)/P1. The higher percentage of metal recovered by P1

compared to the rest of the pine LSs is due to its high carboxylic groups content, and it ionized at pH<7.7 (Yan *et al.*, 2010). In the case of P2, its higher percentage was related to its low organic S content compared to P3 and P5. Thus, P3 could be more suitable for providing Mn and Zn to crops due to its highest complexing capacity and low percentage of metal recovered, which may indicate low stability of these complexes and a better release of micronutrients, according to Benedicto *et al.* (2011).

3.3.5. High-performance size exclusion chromatography of different wood lignosulfonates

The MWD of the pine LS was also analysed by HPSEC, where P2, P1, P3, and P5 presented both HMW and LMW fractions, whereas P4 presented only a LMW fraction (Fig. III.2c), according to the chromatograms obtained by GFC (Fig. III.2b) and those obtained by comparing with the standard LS of known av MW in HPSEC (Fig. III.S3, Supplementary data I). This indicates that the molecular weight distribution depends on the extraction method applied, not only on the plant species isolated (Collins *et al.*, 2019).

The P2 presented the highest percentage of metal recovered for Fe(III)/(II) and Mn (II) compared to the rest of the pine LSs (Table III.3). Thus, it was selected for comparison with eucalyptus LS (E1) and spruce LS (S1) to further study the distribution at pH 6 (to simulate an optimal nutrient solution pH) and pH 9 (to test only the metal complexed by LS) from the three wood species (see chromatograms in Fig. III.3). The P2 (Fig. III.3a and Fig. III.3b) and S1 (Fig. III.3c and Fig. III.3d) presented one fraction that eluted at the exclusion time T_o (~6.1 min), assigned to HMW (>300 kDa), and other fraction that eluted after T_o designed as LMW (<300 kDa). In contrast, E1 (Fig. III.3e and Fig. III.3f) mainly showed a LMW fraction, corroborating its lower MW compared to P2 and S1 (Table III.1). These results are consistent with previous studies (Fredheim *et al.*, 2002; Braaten *et al.*, 2003), where the softwood LS showed a higher MWD (5-398 kDa) compared with hardwood LS. This characteristic of hardwood LS (E1) can promote a better ability to provide micronutrients.

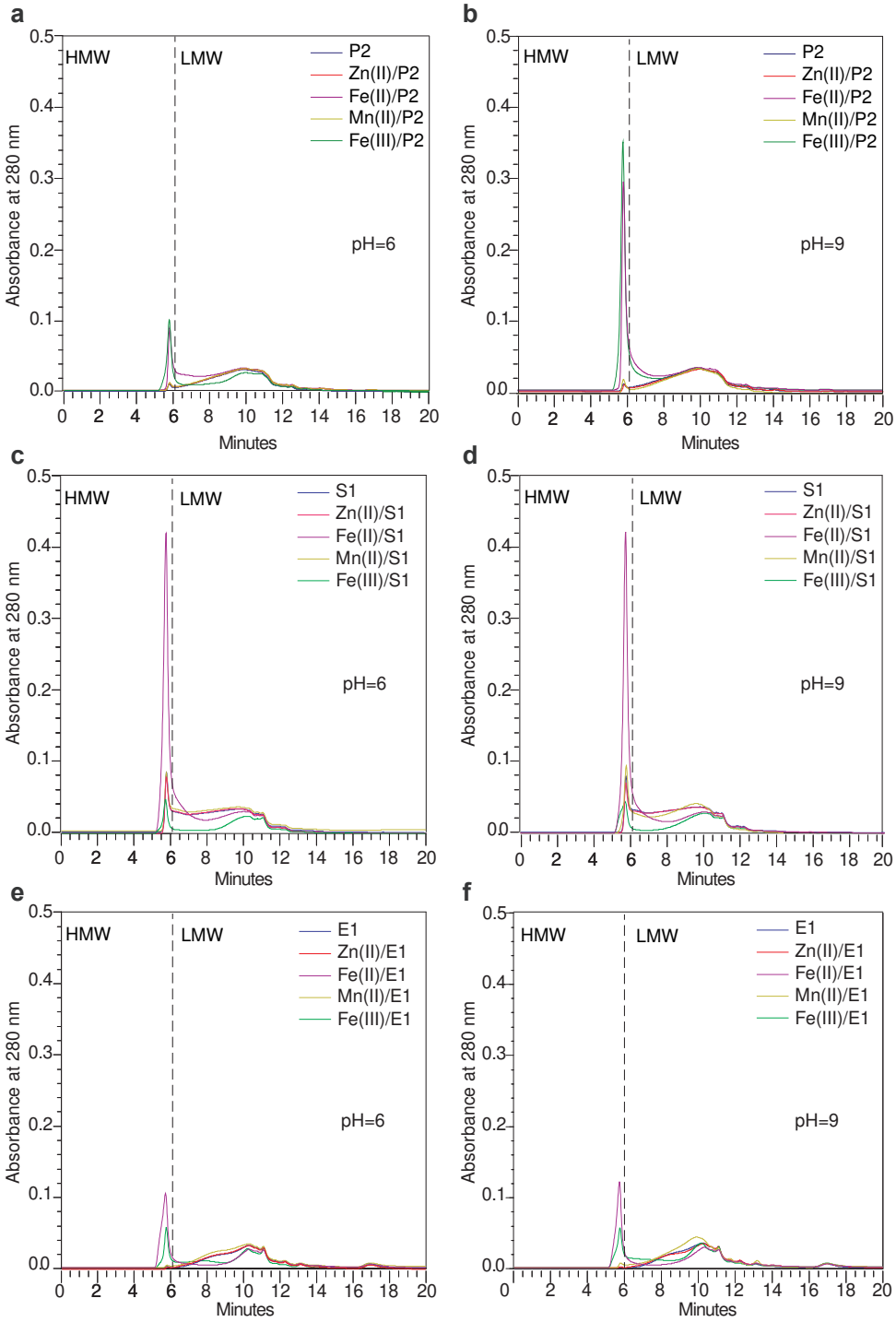

Fig. III.3. Elution profiles obtained by HPSEC on a Biosep Sec-S2000 column using 0.1 M lithium chloride in 90 mM TBAOH. Chromatograms were obtained for the source pine LS (P2) at pH 6 (a) and pH 9 (b) for source spruce LS (S1) and their complexes at pH 6 (c) and 9 (d) and for eucalyptus LS (E1) and their complexes at pH 6 (e) and 9 (f). All LSs were prepared at 0.170% (w/v) and their complexes were formed considering 90% of their MCC. The blue dextran exclusion time is shown as the dotted line.

In the case of complex chromatograms from different wood sources at both pH values (Fig. III.3), all presented a distribution consistent with the results obtained by GFC. Thus, Fe(III) complexes presented an increase in the HMW fraction, indicating the formation of HMW compounds. This effect was less evident for Fe(III)/S1 (Fig. III.3c and Fig. III.3d) and Fe(III)/E1 (Fig. III.3e and Fig. III.4f). Abundant formation of HMW compounds, as a consequence of their higher complexing capacity compared to P2 (Table III.2), was seen, but most were removed by filtration (with a 0.22-µm filter) of the complexes prior to fractionation by HPSEC (Table III.S1, Supplementary data I). Furthermore, Fe(III) complexes showed a higher increase in the HMW fraction at pH 9 than at pH 6, which was supported by the highest Fe content in the filtrate at pH 9 (Table III.S1, supplementary data I). These results suggested a higher concentration of dissolved Fe, which is related to the LS expansion at high pH (Pang *et al.*, 2008), as well as Fe(III) complexation to carboxylic, phenolic, and sulfonic groups that are ionized at alkaline pH, whereas at pH 6 only the sulfonic or carboxylic groups are ionized (Yan *et al.*, 2010). The fingerprint of the Fe(II) complexes confirmed their distribution in both HMW and LMW fractions, as pointed out by the results obtained by GFC (Table III.3). Nonetheless, Fe(II)/S1 (Fig. III.3c and Fig. III.3d) and Fe(II)/E1 (Fig. III.3e and Fig. III.3f) presented a higher increase in the HMW fraction, contrary to Fe(II)/P2 (Fig. III.3a and Fig. III.3b), which did not show correlation with the metal measured in the filtrate (Table III.S1, Supplementary data I). This is associated with the susceptibility of the Fe(II) complexes to oxidation and precipitation (Carrasco *et al.*, 2012) and, consequently, these results were not related to the MCC (Table III.2). Finally, the Zn(II) and Mn(II) complexes presented similar fingerprints to their LS, corroborating that these metals did not induce aggregation.

To further confirm the results of the chromatograms (Fig. III.3), the differences in the percentage of peak area corresponding to the HMW and LMW fractions of the complexes at both pH values were also analysed (Table III.4). As a consequence of these results, the percentage of peak area in the HMW fraction for the Fe(III) complexes was higher at pH 9 than at pH 6 in the following order: P2>E1>S1, according to the chromatograms (Fig. III.3) and the metal measured in the filtrates (Table III.S2, Supplementary data I). These results confirmed the higher complexing capacity of E1 and S1 compared to P2 (Table III.2), related to their higher content of phenolic groups (Table III.1), as was shown by P4 with the highest complexing capacity for Fe(III) compared to the other pine LSs. Therefore, the most abundant HMW compounds are formed from S1 and E1 and retained in the filtrate, contrary to P2.

Consequently, Fe(III)/P2 can be an efficient fertilizer, allowing for a higher concentration of dissolved Fe in the filtrate. The percentage peak area of the Fe(II) complexes decreased in the following order: S1>E1>P2, according to the chromatograms (Fig. III.3). Nonetheless, these results were not related to the MCC (Table III.2) because of the susceptibility of these complexes to oxidation and precipitation (Carrasco *et al*., 2012). Consequently, the LSs are prepared more effectively from ferric salt than ferrous salt, independent of the wood sources or sulphite pulping process from which the LS is obtained. This has been previously observed by Carrasco *et al*. (2012) for E1 and S1, so the weaker complexes are formed by Fe(II) salts.

Table III.4. Distribution of metal based on the percentage peak area obtained by HPSEC between the high molecular weight (HMW) and low molecular weight (LMW) fractions of the complexes at pH 6 and 9 from three different wood species.

	% Peak area at pH 6		**% Peak area at pH 9**	
Sample	HWM	LWM	HWM	LWM
		Pine LS		
P2	3.3	96.7	2.6	97.4
Fe(III)/P2	22.4	77.6	40.5	59.5
Fe(II)/P2	12.8	87.2	29.4	70.6
Zn(II)/P2	2.6	97.4	2.3	97.7
Mn(II)/P2	2.5	97.5	4.1	95.9
		Spruce LS		
S1	11.9	88.1	10.4	89.6
Fe (III)/S1	17.7	82.3	20.0	80.0
Fe (II)/S1	44.1	55.9	45.7	54.3
Zn(II)/S1	10.7	89.3	9.5	90.5
Mn(II)/S1	10.2	89.8	17.4	82.6
		Eucalyptus LS		
E1	0.9	99.1	0.4	99.6
Fe (III)/E1	19.7	80.3	23.3	76.7
Fe (II)/E1	34.8	65.2	31.5	68.5
Zn(II)/E1	0.9	99.1	0.4	99.6
Mn(II)/E1	0.8	99.2	1.5	98.5

The percentage peak area of the Zn(II) complexes (Table III.4) indicated the following order: S1>P2>E1, confirming the higher complexing capacity of S1 compared to E1 obtained in a previous study for Zn (Martín-Ortiz *et al.*, 2009). The lowest percentage peak area presented by Zn(II)/E1 is related to its low stability, according to previous studies (Benedicto *et al.*, 2011; Cieschi *et al.*, 2016), where E1 was able to provide Zn(II) to the plant under calcareous soil and foliar conditions due to its low stability. In the case of Mn(II) complexes, the percentage of peak area decreases in the following order: S1>P2>E1, according to the higher Mn(II) complexing capacity of S1 (Table III.2). Furthermore, the results showed that Mn(II) complexation was pH dependent, which was higher at pH 9. This indicated that S1 could form strong complexes with Mn(II) due to its stability at both pH values (Fig. III.3c and Fig. III.3d) and its higher complexing capacity (Table III.2).

3.3.6. Implications on plant growth development

GFC and HPSEC are useful tools for determining the MWD of Fe, Mn, and Zn LS complexes and for assessing their ability to provide micronutrients to plants. GFC indicated that the amount of metal complexed depends on the sulphite pulping process applied to obtain the LS and on its molecular weight. So, the low molecular weight LS enriched with phenolic groups presented the highest Fe(III) complexing capacity. Fe(III)/LS chromatograms presented a unique fingerprint, which indicated the formation of HMW compounds. Mn(II)/LS and Zn(II)/LS chromatograms presented fingerprints similar to their LSs, suggesting that these metals did not form aggregates. Also, HPSEC was a sensitive and fast method, used to estimate the stability of these complexes. These findings, along with the percentage of metal recovered from GFC and the percentage peak area in the HWM and LMW fractions from HPSEC, have been revealed as important parameters to identify LSs with potential fertilizer capacities and to understand the effectiveness of their complexes as fertilizers. However, the hydroxyl groups and metal/LS ratio should also be considered in the complex stability. So, these parameters can also affect the effectiveness of the complexes, as have previously been reported for S1 and E1 (Rodríguez-Lucena *et al.*, 2011; Carrasco *et al.*, 2012). Thus, bioassays with pine LSs would be necessary to corroborate that they can be a source of biodegradable fertilizers and that they can be used as soil improvers because of their capacity to retain water caused by their network structure (Collins *et al.*, 2019).

IV. Effect of Fe:ligand ratios on hydroponic conditions and calcareous soil *in Solanum lycopersicum* L. and *Glycine max* L. fertilized with heptagluconate and gluconate

Resumen

El riesgo ambiental de la aplicación de quelatos sintéticos ha promovido el uso de complejos biodegradables para corregir la deficiencia de Fe en plantas. En este trabajo, se consideran factores clave para la eficacia de heptagluconato (G7) y gluconato (G6) como fertilizantes: el estado de oxidación del Fe, la relación Fe:ligando y la distribución del peso molecular. Se prepararon y analizaron por cromatografía de filtración en gel (GFC) complejos de diferente relación Fe:ligando. Se evaluó la capacidad de las relaciones Fe:ligando para proveer Fe a plantas de tomate en hidroponía y a plantas de soja en suelo calizo y se comparó con la de quelatos sintéticos (Fe^{3+}:HBED y Fe^{3+}:EDTA). El G7 presentó una mayor capacidad que G6 para complejar tanto Fe(II) como Fe(III), pero los complejos de Fe(II) mostraron baja estabilidad a pH 9 y oxidación en solución. La técnica de GFC reveló la naturaleza polimérica del Fe^{3+}:G7 a diferentes relaciones. La efectividad de los fertilizantes de Fe depende de la relación Fe^{3+}:ligando y el tipo de ligando, siendo las relaciones 1:1 y 1:2 de Fe^{3+}:G7 las más efectivas. La relación 1:1 de Fe^{3+}:G7 también presentó una mejor respuesta en la absorción de otros micronutrientes.

Abstract

The environmental risk of synthetic chelate application promotes the implementation of biodegradables complexes to correct Fe-deficiency in plants. In this paper, the Fe oxidation state, the Fe:ligand ratio and molecular weight distribution for heptagluconate (G7) and gluconate (G6) are consider as key factors for the complexes efficacy as fertilizes. Complexes with different Fe:ligand ratios were prepared and analyzed by gel filtration chromatography (GFC). The ability of Fe:ligand ratios to provide Fe to tomato in hydroponics and soybean in calcareous soil was tested and compared to synthetic chelates (Fe^{3+}:HBED and Fe^{3+}:EDTA). The G7 presented a higher capacity to complex both Fe(II) and Fe(III) than G6, but the Fe(II) complexes show low stability at pH 9 and oxidation in solution. GFC demonstrated the polynuclear nature of the Fe^{3+}:G7 at various ratios. The effectiveness of the Fe fertilizers depend on the Fe^{3+}:ligand ratio and the ligand type being the Fe^{3+}:G7 (1:1 and 1:2) the most effective. The Fe^{3+}:G7 (1:1) also presented a better response for the uptake of other micronutrients.

4.1 INTRODUCTION

Iron (Fe) chlorosis is a nutritional disorder characterized by a decrease of chlorophyll content in leaves. This is a common problem of sensitive crops grown in calcareous soils, since under these conditions; the Fe uptake by the plant is prevented (Marschner, 2012). Iron chlorosis harms several physiological processes such as photosynthesis, chlorophyll biosynthesis, respiration and enzymatic activities (Fodor *et al*., 2012; Ejraei, 2013). Dicotyledonous and non-graminaceous monocotyledonous plants have developed a Fe uptake strategy named Strategy I, inducing the rhizosphere acidification followed by the reduction of Fe^{3+} to Fe^{2+} from membrane-bound enzyme ferric-chelate reductase (Marschner, 2012). Tomato (*Solanum lycopersicum* L.) and soybean (*Glycine max*) are widely used as model plants to investigate the Fe deficiency of the Strategy I (Zamboni *et al*., 2012; Ejraei, 2013). The strategy I plants growing on calcareous soil usually requires the application of synthetic Fe chelates such as the Fe^{3+}:EDTA (ethylenediaminetetraacetate), Fe^{3+}:EDDHA (ethylenediamine-N,N'-bis(hydroxyphenilacetate)) or the Fe^{3+}:HBED (N,N-bis(2-hydroxybenzyl) ethylenediamine-N,N-diacetate), being this last one a highly stable (López-Rayo *et al*., 2009) and effective fertilizer (Nadal *et al*., 2013). Parameters such as the stability of the Fe-chelate and of the chelates formed with the competing ions as Ca^{2+}, the retention on soil surfaces, the plant Fe uptake mechanism (Lucena, 2003) and the so called "shuttle effect mechanism" (Lucena, 2006) affects the effectiveness of Fe-chelates to correct Fe chlorosis. Despite these benefits, synthetic Fe chelates are expensive and may involve environmental risks related to their mobility in the soil (Lucena, 2006). Complexing agents such as sodium gluconate (G6) or sodium glucoheptonate (G7) have a low environmental impact due to their high biodegradability (Maxwell, 2004). They can complex metals through their carboxylic and hydroxylic groups by different binding modes depending on the metal itself and the reaction conditions. Only a few studies have explored the effect of G6 and G7 in plant nutrition, with contradictory results. An adequate capacity of the Fe^{3+}:G6 (1:2) to correct chlorotic soybeans grown in calcareous soil was observed by Martín-Fernández *et al*. (2017a) similar to Fe^{3+}:IDHA (Iminodisuccinate), and Clemens *et al*. (1990) suggested that the G7 would be a better complexing agent for Fe^{3+} than EDTA and G6 in alkaline soils. Also, Fuentes *et al*. (2018) in a gene expression study in Fe-deficient cucumber plants found that the foliar application of Fe^{3+}:G7 was effective providing Fe, but its delivering was slow or scarce. On the contrary, Shaddox *et al*. (2016) reported a decrease of soluble Fe after one-day application of the Fe^{2+}:G7 in incubated soils similar to ferrous sulfate. Similar results were obtained by Goos and Germain (2001)

when Fe^{3+}:G7 and Fe^{3+}:G6 were applied to soil in batch incubation experiments in comparison with EDTA, DTPA (diethlyenetriaminepentaacetic), EDDHA and EDDHSA (ethylendiamino-N'N-bis (2-hydroxy-5-sulfo) phenylacetic acid). Thus, the effect of Fe complexes of G6 and G7 on plant nutrition is limited or not conclusive.

Most of these studies were conducted by using a 1:1 and 1:2 (Fe:ligand) molar ratio. However, the metal complexes can form a wider variety of chemical species, depending on the ligand type, pH, the Fe:ligand molar ratio and synthesis temperature, affecting to the effectiveness of the complexes. These effects have been observed for complexes of several natures. For instance, Fe^{3+} forms very stable complexes in solution with ligands such as sugars and citrate due to the formation of polymers, preventing the precipitation of the Fe(III)-hydroxy-polymer (Spiro *et al*., 1967; Tonković *et al*., 1983). Studies conducted by Silva *et al*. (2009) with Fe citrate complexes, demonstrated that at pH 9, a mononuclear Fe complex is predominant with a low Fe^{3+}:citrate molar ratio, whereas a high Fe^{3+}:citrate molar ratio at neutral pH lead to the formation of oligomeric complexes. Stevenson (1982), showed that humic metal complexes with high metal:humic acid ratios presented a lower stability than those with low metal:humic acid ratios. Experiments conducted with lignosulfonates and humic acid from leonardite with different iron sources [Fe (III) and Fe (II)] confirmed the effect of the Fe:ligand ratios in the binding sites and the complexes stability. The Fe source was also determinant in the formation of weak or strong complexes (Carrasco *et al*., 2012; Kovács *et al*., 2013), indicating that strong complexes are prepared from the Fe^{3+}. It is also remarkable that an excess of chelating agents in nutrient solutions can retard, and even inhibit, the uptake of metals by plants (Lindsay and Schwab, 1982; Wallace *et al*., 1983). Then, a better knowledge of the effect of the Fe:ligand ratio with G6 and G7 complexes plays an important role to study their efficacy as Fe fertilizers and their possible "shuttle effect" for the turnover of micronutrients naturally present in the soil solution, similar to that already demonstrated for synthetic chelating agents.

Currently, several spectroscopic techniques such as ^{13}C nuclear magnetic resonance (^{13}C-NMR), Fourier transformed infrared (FTIR) and Mössbauer spectroscopy are considered to determine some physical characteristics of the metal complexes such as the purity, geometry and structure, the bonding sites and the Fe oxidation states. Besides, gel filtration chromatography (GFC) on Sephadex® has demonstrated to be a useful tool for the characterization of metal-complexes and metal:chelates. The GFC permits the fractionation based on size (Tonković *et al*., 1983; Pérez-Sanz *et al*., 2006) and also the identification of the free and complexed metal by comparison between the retention time and the quantification of the soluble Fe in the obtained fractions

(Boxema, 1979; Deacon *et al.*, 1993). By the application of this technique, the presence of polynuclear compounds in Fe^{3+}:G6 (1:2) complexes (Martín-Fernández *et al.*, 2017a), and low molecular compounds in Fe^{2+}:G6 (1:2) (Nikolić *et al.*, 2014) could be determined. To understand, the discrepancies obtained in previous studies for the molecular weight distribution of Fe complexes, which may be related to the Fe:ligand ratio and the Fe source used.

Although a few studies have proved the efficacy of the G6 and G7 Fe complexes to correct Fe chlorosis, their effectiveness related to parameters such as Fe:ligand ratio, molecular weight distribution, and the Fe complexing capacity with different Fe sources has not been explored. Therefore, it can be hypothesized that the Fe:ligand molar ratio of G6 and G7 is an important parameter for their effectiveness as Fe fertilizers in plants under hydroponics and calcareous soil. For that purpose, firstly, the complexing capacity of the complexing agents G6 and G7 with a different Fe source [Fe (III) and Fe (II)] was evaluated to improve the stability of the complexes and the amount of Fe provided to the plants. Secondly, the molecular weight distribution of the Fe:ligand molar ratios obtained were chemically characterized. Finally, the effectiveness of the complexes provides in different Fe:ligand ratios to supply Fe to strategy I plants under hydroponic and calcareous soil conditions was evaluated.

4.2 MATERIALS AND METHODS

Pure reagents of sodium gluconate (Sigma Aldrich, ≥99%) assigned as G6, and α−sodium glucoheptonate dihydrate (G7) kindly provided by DABEER (99%, Barcelona, Spain) were used. Synthetic chelating agents of ethylenediaminetetraacetic and disodium salt [Na_2EDTA, tritriplex III (Merck, 99%)] and N-N´-bis (2-hydroxybenzyl) ethylenediamine-N-N´-diacetic acid (HBED, 93.72%) provided by ADOB PPC; Poznan, Poland were used for comparison. Iron complexes were prepared with $FeCl_3 \cdot 6H_2O$ (Merck, 99%), or $FeSO_4 \cdot 7H_2O$ (Merck, 99%) of analytical grade and the water used was grade I (ISO 3696, 1987), free of organic contaminants.

4.2.1 Complexing capacity of gluconate and heptagluconate and Fe complexes preparation

The maximum complexing capacity (MCC) of G6 and G7 with Fe (III)/Fe (II) was determined by the maximum Fe content remaining in solution at pH 9 after one day in the dark according to the method previously described by Villén et al. (2007).

After that, Fe complexes were prepared at room temperature with the aim to prepare several Fe:ligand molar ratios according the dry weights of the ligands: 1:0.5 for G7 with Fe^{3+}, and 1:1, 1:2 and 1:3 (Fe:ligand molar ratios) for both G6 and G7 with Fe^{3+}, and 1:2 for G6 with Fe^{2+}, and 1:1 and 1:2 (Fe:ligand molar ratios) for G7 with Fe^{2+}. These complexes were selected based on the stability observed during the determination of the MCC, being the Fe^{3+} complexes the most stable and the Fe^{2+} the less. The complexes were prepared at pH 6-7 and then freeze-dried. The total soluble Fe in the freeze-dried samples was determined following the methods 9.2, 9.3 and 9.4 (EU, 2003) by extraction with water and filtration through a 0.45 µm filter. The total complexed Fe in the samples was analyzed according to EN 15962:2011 (CEN, 2011), following the same preparation then that for the soluble element but rising the pH to 9 before the filtration. In addition, the complexed fraction (expressed as percentage of complexed Fe with respect to the soluble Fe) was determined as an index of the complex stability and its effectiveness as fertilizer (Rodríguez-Lucena *et al.*, 2010). A flame atomic absorption spectrometer (AAS, Perkin-Elmer AAnalyst 800; Shelton, CT, USA) was used for all the Fe determinations.

4.2.2 Structural characterization of the complexing agents and the Fe complexes

The structural changes of G6 and G7 after complexation with Fe^{3+} in the different ratios G6 (1:1) and G7 (1:0.5, 1:1, 1:2 and 1:3) were analyzed by FTIR spectra on a Bruker IFS66vd spectrometer (Germany) using KBr pellet method in the 3800-600 cm^{-1} region at a resolution of 4 cm^{-1} in the transmittance mode. ^{13}C-NMR spectra of G6 and G7 dissolved in deuterated water (D_2O) were recorded on a Bruker DRX 500 MHz (Germany) to check their chemical structure. ^{57}Fe Mössbauer spectroscopy was used to analyze the oxidation state of Fe of G7 complexes prepared with Fe^{2+} or Fe^{3+} (at 1:2 molar ratio). The analysis was conducted at T= 298 K in triangle mode with a ^{57}Co (Rh) source with and activity of 50 mCi and calibrated with 6 µm α-iron at room temperature. The principal parameters obtained from the spectra were isomer shift (δ, mm s^{-1}), quadrupole splitting (Δ, mm s^{-1}), and relative content area (A, %), which can provide information about the oxidation state, the coordination number of the resonant nucleus and the semiquantitative information for the species obtained.

4.2.3 Gel filtration chromatographic

The molecular weight distribution of the Fe^{3+}:G7 complexes were analyzed on a glass column (1.0 x 30 cm) packed with Sephadex® G-10 (molecular weight cut-off, MWCO >700 Da; 40-120 µm particle size distribution from Sigma Aldrich). The Fe^{3+}:G7 (1:3) was also analysed by a Sephadex® G-15 (MWCO >1500 Da; 40-120 µm particle size distribution). The Fe^{3+}:G7 complexes were additionally eluted in a Sephadex® G-25 (MWCO >5000 Da; 50-150 µm particle size distribution). The samples were dissolved in 0.6 % (w/v) of 0.1 M NaCl at pH 6, filtrated by 0.22 µm filter, and the Fe content measured by AAS prior to the fractionation under gravity of 125 µL in the columns at room temperature. The fractions were monitored at 220 nm in a Spectrostar nano microplate reader (BMG Labtech, Ortenberg, Germany) and the Fe concentration analysed by AAS after gel filtration chromatography on Sephadex® G-10. The exclusion volume (V_o) was determined with blue dextran 2000 (MW~2000 kDa) and the total volume (V_p) with Fe^{3+}:HBED (MW~440 Da) from 0.1 M NaCl at pH 6. In agreement with a previous study in which the molecular weight distribution of Fe:lignosulfonate complexes were evaluated (Islas-Valdez *et al*., 2019).

4.2.4 Plant experiments

Two experiments were conducted by using two different Strategy I plants sensitive to Fe chlorosis. Tomato was grown under hydroponics and soybean was grown in calcareous soil conditions. Both experiments were done in a growth chamber (Dycometal type CCK) with a photoperiod of 16 h to 23ºC and 40% relative humidity during the light period, and 8 h to 19ºC and 60% relative humidity during the dark period. The composition of the full-strength nutrient solution (NS) was: macronutrient (mM) 1.0 Ca $(NO_3)_2{\cdot}4H_2O$, 0.9 KNO_3, 0.3 $MgSO_4{\cdot}7H_2O$, 0.1 KH_2PO_4; micronutrients (µM) 2.5 $MnSO_4{\cdot}H_2O$, 1.0 $CuSO_4{\cdot}5H_2O$, 10.0 $ZnSO_4{\cdot}7H_2O$, 1.0 $NiCl_2{\cdot}6H_2O$, 1.0 $CoSO_4{\cdot}7H_2O$, 115.5 Na_2EDTA, 35.0 NaCl, 10.0 H_3BO_3, 0.05 $Na_2MoO_4{\cdot}2H_2O$.

4.2.4.1 Growth conditions in the hydroponic experiment

Tomato (*Solanum lycopersicum* L., cv. Marmade) seeds were germinated for 12 days in vermiculite (1-4 mm grain; Projar, Spain) moistened with 1mM $CaSO_4$. Uniform seedlings were transferred to 1.8 L vessels filled with 1/4 diluted NS for three days containing 5 µM Fe^{3+}:HBED at pH 6, and then, 11 more days in full strength NS at pH 7.5. After that, they grow for eight days in a Fe-free full-strength NS. The plants were

individually transferred to 250 mL vessels containing the NS and the Fe treatments. In this experiment, the Fe^{3+}:G7 complexes at several molar ratios were studied and compared to the synthetic chelate Fe^{3+}:HBED as the positive control, assayed in a concentration of 10 µM of Fe^{3+}. This low Fe concentration permits a better differentiation of the effect in the Fe nutrition between the treatments (Lucena and Chaney, 2007). Likewise, a Fe-free negative control (-Fe) was also assayed for comparison. The nutrient solution was continuously aerated and buffered at pH 7.5 with 1.0 x 10^{-4} M HEPES and 0.1g L^{-1} of $CaCO_3$ to simulate calcareous soil conditions. The sampling was done 15 days after the treatment (DAT). The NS was renewed every seven days.

4.2.4.2 Growth conditions in the soil experiment

Soybean *(Glycine max* L., cv. RGT Speeda) seeds were germinated in perlite (1-5 mm grain; Projar, Spain) moistened with distilled water for 12 days. Uniform seedlings were transferred to 4 L vessels filled with a 1/5 diluted NS containing 10 µM Fe^{3+}:HBED at pH 6 for seven days and for seven more days in a Fe-free full strength NS. After this pre-growth period under hydroponic conditions, two seedlings per pot were transplanted to polystyrene pots covered with aluminium foil to avoid photodegradation of Fe complexes (Hernández-Apaolaza and Lucena, 2011), filled with 180 g calcareous sand (975 g Kg^{-1} $CaCO_3$; 2-4 mm) mixed with 420 g of a sandy loam soil (pH 7.9; 435 g Kg^{-1} sand, 80 g Kg^{-1} silt, 485 g Kg^{-1} clay; 9.2 g Kg^{-1} organic matter; 380 g Kg^{-1} total $CaCO_3$, 89 g Kg^{-1} active lime; and Soltanpour and Schwab (1977) extractable micronutrients: 5.3 g Kg^{-1} Fe, 4.5 g Kg^{-1} Mn, 1.0 g Kg^{-1} Cu and 3.0 g Kg^{-1} Zn) from Picassent (Valencia, Spain). This soil has been previously described (Martín-Fernández *et al.*, 2017a).

Two days before transplanting, six pots per treatment were irrigated until 100% of the soil-sand mixture water holding capacity (SWHC). Two days after, the treatments were initiated: the Fe^{3+}:G6 (1:1), the molar ratios of Fe^{3+}:G7 (1:0.5, 1:1, 1:2 and 1:3) and, the Fe^{3+}:EDTA (positive control). The solutions of the Fe complexes and the Fe^{3+}:EDTA were split over the experiment: 0, 7 and 14 after the first treatment application at an Fe dose of 4.2, 2.1, and 2.1 µmol Fe^{3+} per pot, respectively. In addition, a Fe-free negative control (-Fe) was assayed. During the experiment, pots were irrigated until 80% SWHC every two or three days with a macronutrient NS in 0.1g L^{-1} of $CaCO_3$. One plant shoot per pot was sampled at 7 DAT and grouped in three pairs per treatment (three analytical replicates). At 21 DAT, the remaining plants shoots in each pot and the roots were sampled and kept separated in six replicates per treatment. On completion of the

experiments, the soluble and available Fe, Mn, Zn and Cu fractions in soil were determined by the extraction method proposed by Nadal *et al*. (2009) with water and DTPA solutions (Soltanpour and Schwab, 1977) followed by the acidification with HNO_3 (65%, Merck) to 1% and analyzed by AAS.

4.2.5 Physiological parameters

Leaf chlorophyll index was measured every two or three days after the beginning of the treatments on the youngest and fully expanded leaf (three readings per level), by using a portable chlorophyll meter Dualex 4 Scientific (FORCE-A, Orsay, France). Shoot and root lengths were measured after each sampling. Leaves, stems and roots were separated and washed with 0.1% non-ionic detergent (Tween 80) and 0.1M HCl followed by tap-water and distilled water (Álvarez-Fernández *et al*., 2001), and finally wiped and weighed to obtain the fresh weight. Plant tissues were dried in a forced air oven at 65 ºC for three days until constant weight to obtain dry weight (DW) and ground with a porcelain mortar and pestle. Samples were mineralized by dry digestion in a muffle furnace at 480 ºC for 4 h followed by the acid digestion with HCl suprapur (1:1) for the ash solubilization at 80 ºC for 30 min (Jones, 2001). Total Fe, Mn, Cu and Zn concentration in the plant tissues extracts was determined by AAS.

4.2.6 Statistical analysis

Data were analysed by using the IBM SPSS statistical software (version 23.0; SPSS Inc., Chicago, IL, USA). Differences among treatments were tested by one-way analysis of variance (ANOVA), with a Duncan post hoc test at *p-value* < 0.05.

4.3. RESULTS

4.3.1 Fe complexing capacity of gluconate and heptagluconate

The Fe MCC complexing capacity of Fe^{3+}:G7, Fe^{2+}:G7, Fe^{3+}:G6 and Fe^{2+}:G6 (Fig. IV.1), was determined by the intersection point between the complexing segment and the coagulation segment, as a consequence of the excess of metal. Here, the highest values of the MCC for Fe^{3+} and Fe^{2+} were obtained by G7 (2.65 and 1.25 mol Fe mol^{-1} ligand, respectively), and the lowest for G6 (1.85 and 0.47 mol Fe mol^{-1} ligand, respectively).

4.3.2 Soluble and complexed Fe of the complexes at the different Fe:ligand ratios

Because of the highest complexing capacity presented by G7 with Fe^{3+}, a wider range of molar ratios was prepared for this complex (1:0.5, 1:1, 1:2 and 1:3) for further analysis. In addition, the equimolar ratio of Fe^{3+}:G6 was studied since it has been previously evaluated in other studies. As a result of the low complexing capacity presented by G7 and G6 with the divalent Fe^{2+}, only the 1:1 and 1:2 molar ratios for Fe^{2+}:G7, as well as the 1:2 molar ratio for Fe^{2+}:G6 were further explored. The freeze-dried samples of the Fe^{2+} complexes presented a pale green color while the Fe^{3+} complexes a yellow color. However, after dissolution of the solid samples, both Fe^{2+} and Fe^{3+}complexes presented a yellow color, which suggest the oxidation of the Fe^{2+} complexes.

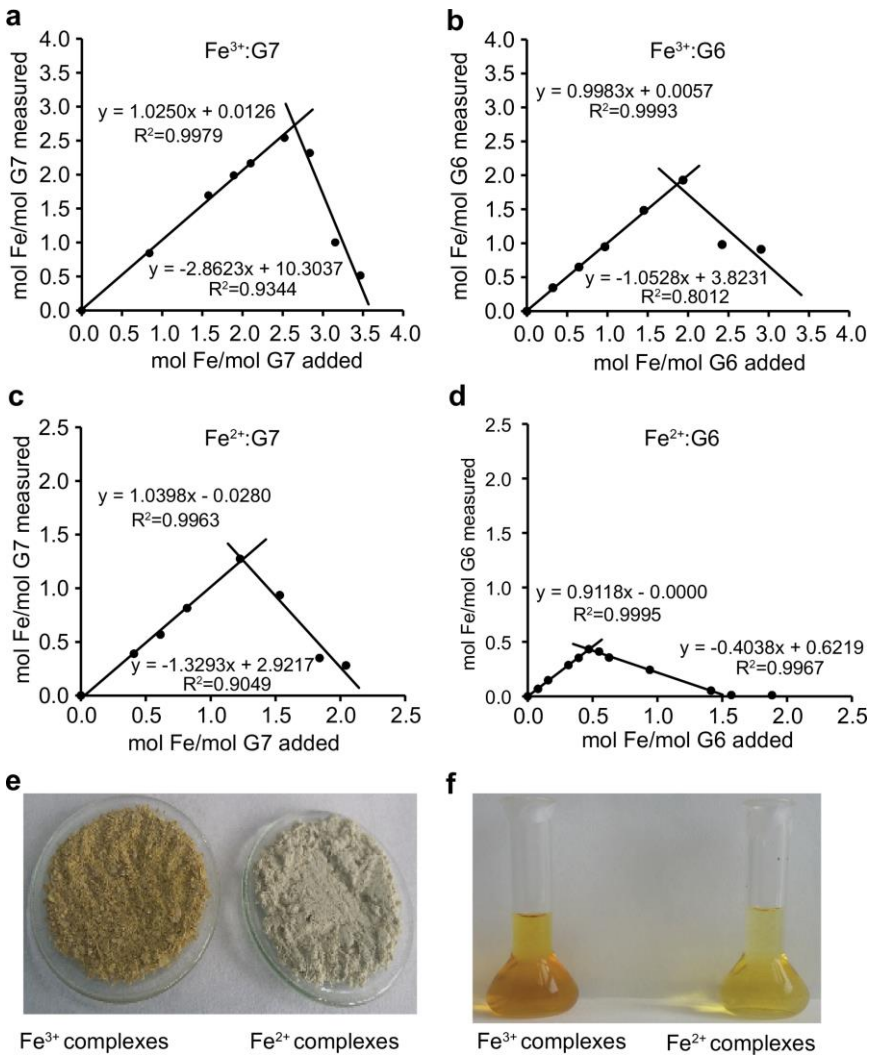

Fig. IV.1: Titration curves for the determination of the maximum complexing capacity of heptagluconate (G7) with a) Fe^{3+} and c) Fe^{2+}, and gluconate (G6) with b) Fe^{3+} and d) Fe^{2+}.

The results obtained for the analysis of the soluble Fe in the above mentioned prepared complexes were checked with the European official method for fertilizers (EU, 2003). Accordingly, the complexes of Fe^{3+}:G7 (1:0.5, 1:1, 1:2 y 1:3), Fe^{3+}:G6 (1:1), Fe^{2+}:G7 (1:1 y 1:2) and Fe^{2+}:G6 (1:2) presented 14, 11, 9.1, 5.8, 11, 9.9, 6.7 and 6.2%, respectively. Then, all of them complied with the minimum percentage of the soluble Fe required by the regulation (EU, 2003) (5% for solid samples). Attending to the fraction of Fe complexed (CEN, 2011), a minimum of 80% is required (EU, 2003). The Fe^{3+}:G7 (1:1), Fe^{3+}:G7 (1:3), Fe^{2+}:G7 (1:1) and Fe^{2+}:G6 (1:2) products presented higher values (86, 100, 90 and 89%, respectively), but Fe^{3+}:G7 (1:0.5), Fe^{3+}:G7 (1:2), Fe^{3+}:G6 (1:1) and the Fe^{2+}:G7 (1:2) (2.0, 74, 77 and 74%, respectively) lower than the 80% required.

4.3.3 Structural characterization of the complexing agents and Fe complexes

The ^{13}C NMR spectra of the G6 and G7 free ligands presented six and seven, respectively, well-defined peaks in the range of 62.61-178.59 ppm (Fig. IV.2), suggesting that both products contained the pure complexing agent. This fact was confirmed by the FTIR spectra of G6 and G7, presenting the G6 a similar spectrum to the sodium gluconate previously reported (Nikolić *et al.*, 2014). The FTIR spectra of Fe^{3+}:G6 (1:1) and the different molar ratios of Fe^{3+}:G7 were also analyzed. In all of them, a broad band at 3400 cm^{-1} due to the vibration of hydroxyl groups, bands at 1625 and 1380 cm^{-1} assigned to asymmetric and symmetric carboxylate anion were observed. The C-O vibration of the primary and secondary hydroxyl groups appeared at 1057 cm^{-1} and 1090 cm^{-1}, respectively (Nikolić *et al.*, 2014). In addition, bands at 2943-2893 cm^{-1} were associated with C-H stretching vibration (Tonković *et al.*, 1983) and the vibrations of Fe-O were presented in the region 1000-600 cm^{-1}. A weak band was additionally presented by Fe^{3+}:G7 (1:2) at 1778 cm^{-1}, which indicated the dissociation of the carboxylic group (Tonković *et al.*, 1983) involved in the Fe complexation.

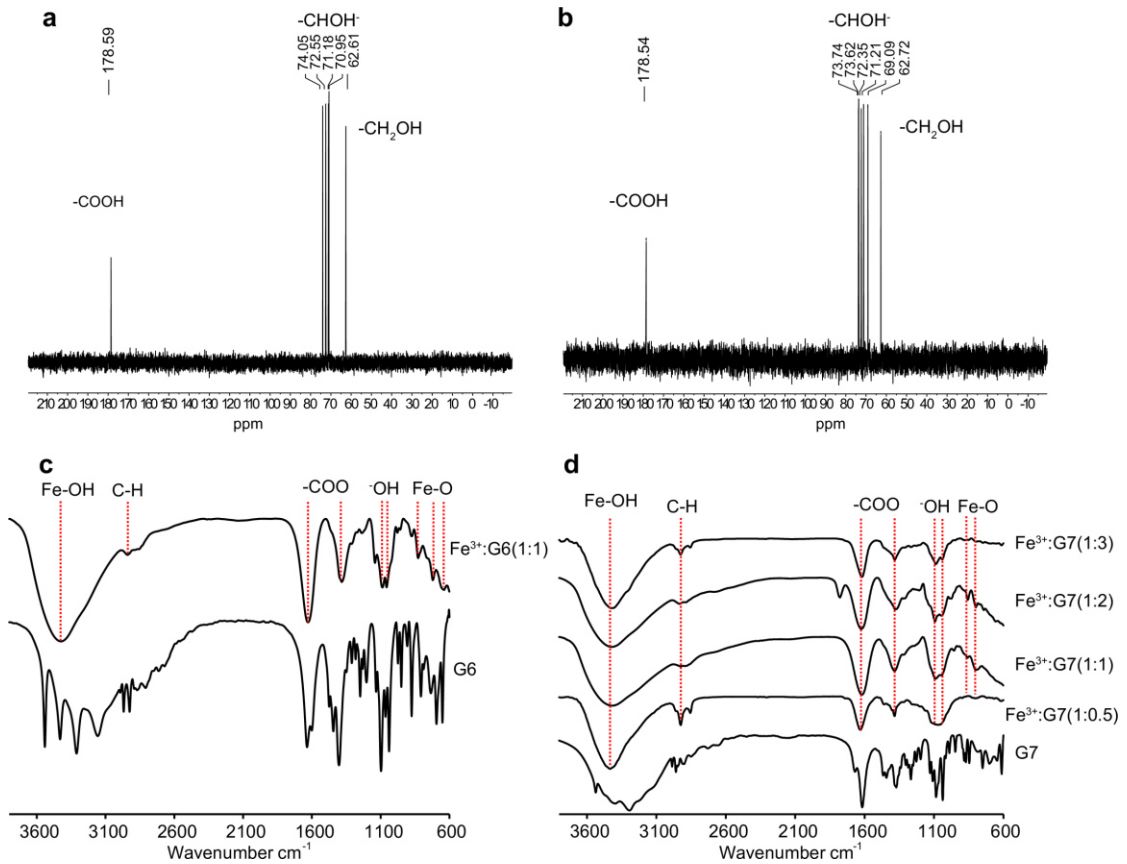

Figure IV.2: ^{13}C-NMR spectra of a) G6 and b) G7. FTIR spectra of c) G6 and Fe^{3+}:G6 (1:1) and d) G7 and the Fe^{3+}:G7 complexes.

To confirm the oxidation state presented in the final complexes, Fe^{2+}:G7 (1:2) and Fe^{3+}:G7 (1:2) were selected and further analyzed by Mössbauer spectroscopy. The Fe^{3+}:G7 (1:2) spectrum reflects two symmetrical doublets (Fig. IV.3a). The main component denoted by Fe^{3+}_A with δ= 0.37 mm s^{-1}, Δ= 0.75mm s^{-1} and *a*= 56% represents a high spin Fe^{3+} that can be associated with ferrihydrite. The Fe^{3+}_B has δ= 0.38 mm s^{-1}, Δ= 1.22 mm s^{-1} and *a*= 44% compatible with Fe^{3+} polynuclear structures (Cieschi *et al.*, 2017). Attending to Fe^{2+}:G7 (1:2) spectrum (Fig. IV.3b) reflects an asymmetric doublet by the superposition of the Fe^{2+} (δ=1.25 mm s^{-1}, Δ=2.51 mm s^{-1} and a=76%) and Fe^{3+} (δ= 0.26 mm s^{-1}, Δ= 1.10 mm s^{-1} and a=24%) phases, respectively. The Fe^{2+} phase is in good agreement with those of high-spin Fe^{2+} in a distorted octahedral O^6 coordination (Kovács *et al.*, 2013), which may indicate bind of the Fe^{2+} with carboxylic and hydroxylic groups of the G7. The Fe^{3+} phase is also related to iron polynuclear structures.

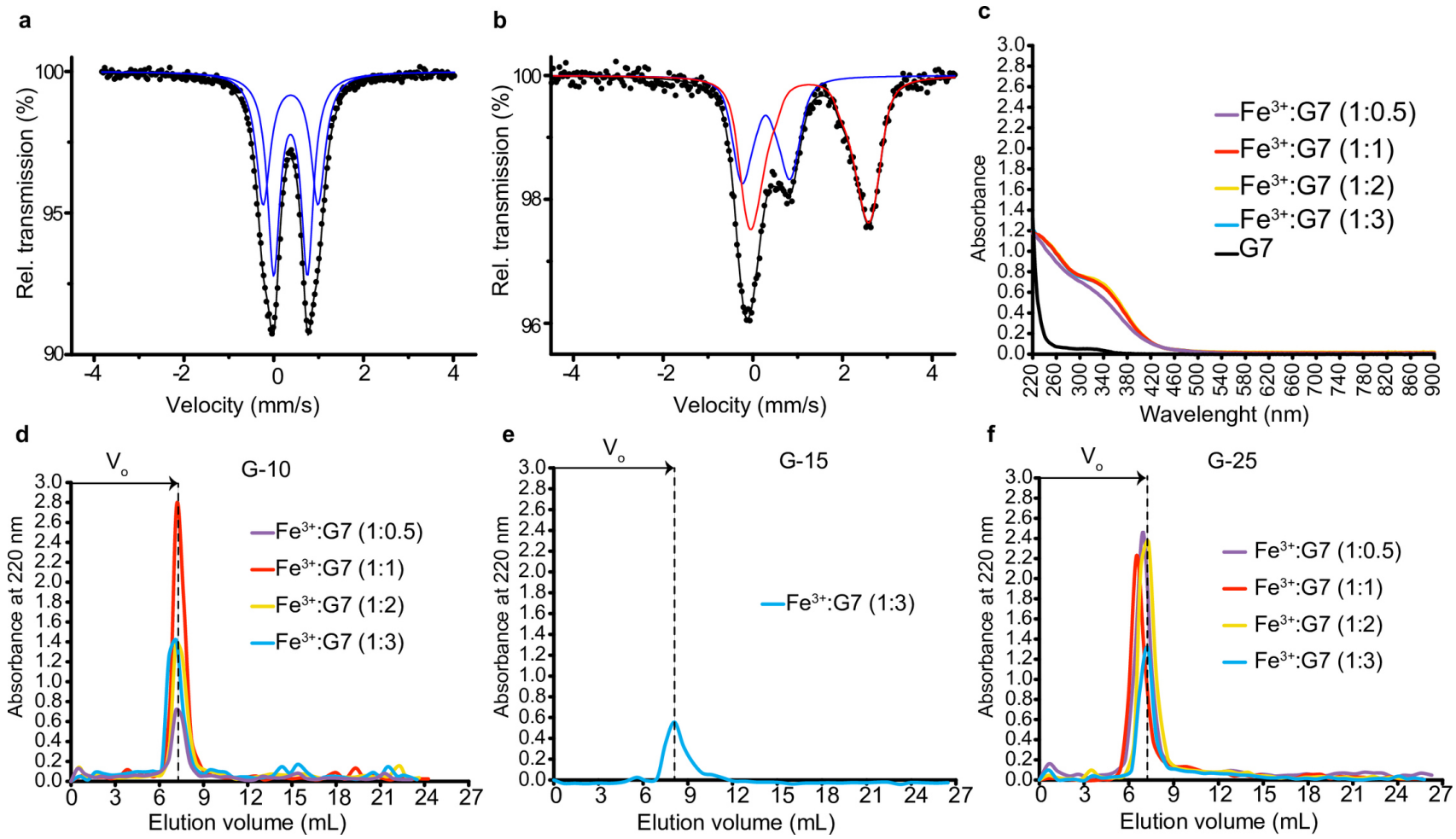

Figure IV.3: ^{57}Fe Mössbauer spectra of a) Fe^{3+}:G7 (1:2) and b) Fe^{2+}:G7 (1:2) recorded at 298 K. The blue line indicates Fe^{3+} and the red line indicates Fe^{2+}. c) Absorption spectra of the molar ratios of Fe^{3+}:G7 and G7. d) Gel filtration chromatography of the Fe^{3+}:G7 complexes eluted on Sephadex® G-10. e) Elution of Fe^{3+}:G7 (1:3) on Sephadex® G-15 and f) elution of the molar ratios of Fe^{3+}:G7 on Sephadex® G-25 with 0.1 M NaCl at pH 6. V_o indicate the exclusion volume marker in each column.

As mentioned above, the Fe^{2+} complexes oxidized after dissolution and the G6 complexes presented lower complexing capacity, thus the different molar ratios were only prepared for the Fe^{3+}:G7 complexes. These complexes were analyzed by UV absorption spectroscopy (Fig. IV.3c), showing a strong band about 350 nm due to the oxo-metal charge transfer absorption band. This band is characteristic of the Fe^{3+} complexes in a high spin state octahedrally chelated by oxygen, as it has been reported for Fe^{3+}:gluconate with an approximate molecular weight 11.6 kDa (Kudasheva *et al.*, 2004).

Concerning the molecular weight distribution of the Fe^{3+}:G7 complexes with different molar ratios on Sephadex® G-10, all eluted in the exclusion volume similar to the blue dextran 2000 eluting at 7.2 mL, suggesting the formation of complexes with a molecular weight higher than 700 Da (Fig. IV.3d). This fact was confirmed by the high percentage of Fe recovered in the peak exclusion with values about 88%, 75%, 70% and 85% for Fe^{3+}:G7 (1:0.5, 1:1, 1:2 and 1:3 molar ratios, respectively) of the total content of Fe eluted. In addition, Fe^{3+}:G7 (1:3) was also passed through the Sephadex® G-15 (Fig. IV.3e) and eluted in the exclusion volume (8.1 mL), indicating the formation of a complex with a molecular weight higher than 1500 Da. Finally, all samples with the different molar ratios were also passed through the Sephadex® G-25 (Fig. IV.3f), as well as those which also eluted in the exclusion volume (8.4 mL), indicating a molecular weight higher than 5000 Da. These results confirmed the tendency of the Fe^{3+} complexes to form polynuclear compounds

4.3.4 Effect of the Fe complexes on tomato seedlings under hydroponic conditions

The evolution of the chlorophyll index of the youngest, fully expanded leaf (fifth leaf level) by Dualex is presented in (Fig. IV.4). The -Fe treatment suffered a detriment of the chlorophyll index over the experiment, showing visible symptoms of Fe deficiency such as the yellowing of the fully expanded leaves and the proliferation of lateral roots. In contrast, all the Fe treatments showed a recovery of the chlorophyll index from 6 DAT with a leaf re-greening. All the molar ratios of the Fe^{3+}:G7 complexes presented similar chlorophyll indices (29.5-30.5) than the positive control Fe^{3+}:HBED (31.9) at 15 DAT.

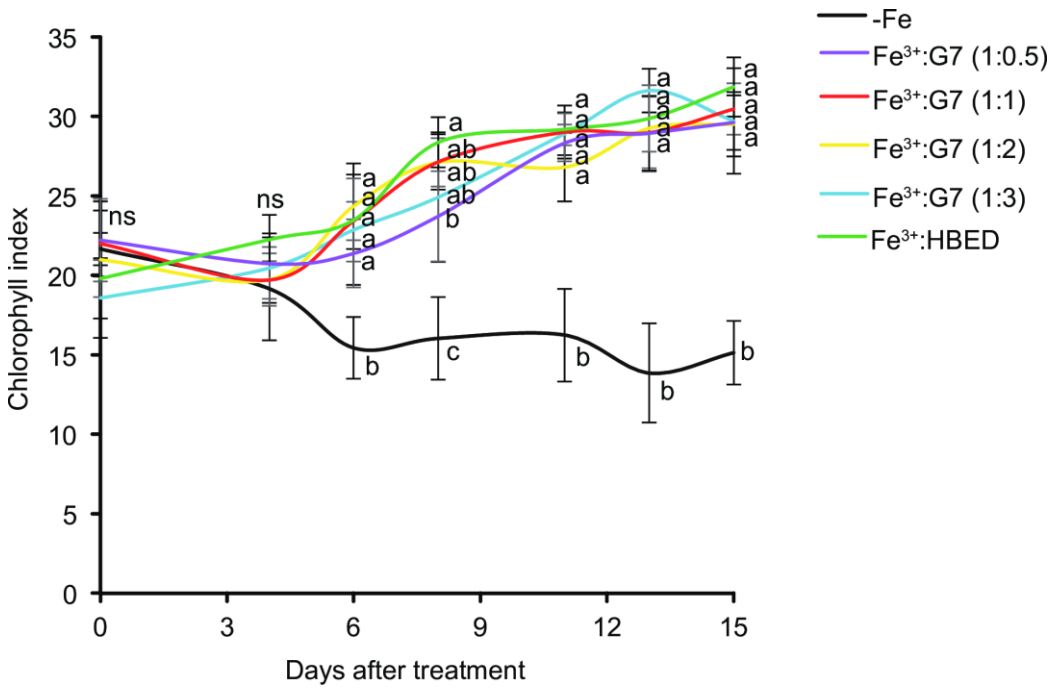

Figure IV.4: Effect of the treatments applied to tomato plants under hydroponic conditions on a) chlorophyll index measured by Dualex in the fifth leaf level. Error bars indicate the standard error (N=5). Different letters represent significant differences among treatments following Duncan test ($p \leq 0.05$ level). ns, not significant.

No differences in the shoot length among treatments were observed at the end of the experiment (15 DAT) (Table IV.1). The application of the Fe^{3+}:G7 (1:1) and the Fe^{3+}:G7 (1:2) increased significantly the DW (by 0.3- and 0.2-fold, respectively) and the elongation of the root (by 0.5- and 0.6-fold, respectively) compared to the Fe^{3+}:HBED. The lowest values were obtained for the –Fe treatment with a 0.8-fold lower than the Fe^{3+}:HBED. A similar effect of the different molar ratios of Fe^{3+}:G7 to Fe^{3+}:HBED on the Fe concentration in leaf was obtained. They presented an Fe concentration 1.5-fold higher than the –Fe treatment, indicating a clear Fe deficiency at 15 DAT. Although, the Fe treatments increased the Fe concentration in stem compared to the Fe-deficient plants, the highest Fe concentration was obtained by Fe^{3+}:HBED with a 2-fold increase. In roots, the Fe concentration was clearly lower in the -Fe treatment. Here, the Fe^{3+}:HBED presented the lower value of the Fe treated plants; similar Fe concentrations were obtained by the Fe complexes at different ratios except for the 1:0.5 ratio which presented the highest, raising 1.3-fold as compared to the Fe^{3+}:HBED treatment. Furthermore, the Fe translocation (TR) was calculated as the percentage of Fe in leaf per Fe in the root. The highest Fe TR was presented by the Fe^{3+}:G7 (1:1), similar to the Fe^{3+}:HBED, but the lowest was obtained by the Fe^{3+}:G7 (1:0.5) with a 1.2-fold decrease compared to the Fe^{3+}:HBED.

Table IV.1: Data of growth, Fe concentration and translocation (TR, leaf concentration/root concentration) in tomato plants at 15 DAT, grown under hydroponic conditions.

Treatment	Length (cm)		DW (g plant^{-1})		Fe (mg Kg^{-1} DW)			Fe TR (%)
	Shoot	Root	Shoot	Root	Leaf	Stem	Root	Leaf/root
-Fe	22.8^{ns}	24.8^{c}	0.97^{c}	0.19^{c}	19.0^{b}	9.48^{e}	32.1^{d}	55.4^{b}
Fe^{3+}:G7 (1:0.5)	25.5	40.0^{b}	1.75^{b}	0.25^{c}	54.4^{a}	20.7^{d}	152^{a}	34.4^{c}
Fe^{3+}:G7 (1:1)	24.3	53.3^{a}	1.81^{b}	0.46^{ab}	59.5^{a}	31.4^{bc}	104^{b}	65.5^{ab}
Fe^{3+}:G7 (1:2)	24.1	48.3^{ab}	2.41^{a}	0.49^{a}	50.3^{a}	26.7^{c}	102^{b}	58.4^{b}
Fe^{3+}:G7 (1:3)	27.5	40.1^{b}	1.62^{b}	0.33^{bc}	55.2^{a}	32.9^{b}	101^{b}	58.6^{b}
Fe^{3+}:HBED	24.8	42.6^{b}	2.01^{ab}	0.33^{bc}	49.1^{a}	38.5^{a}	61.1^{c}	81.8^{a}

Different letters in the same column indicate significant differences among treatments according to Duncan´s test ($p \leq 0.05$). ns, not significant.

In addition, the influence of the treatments in other metal micronutrient concentrations was studied (Table IV.2). The –Fe treatment presented the highest values for Mn, Zn and Cu in some tissues as compared to the Fe treatments. A similar increase was obtained for the Mn concentration in leaf and stem by Fe^{3+}:G7 (1:1) and Fe^{3+}:G7 (1:2), respectively. The Fe^{3+}:HBED treatment showed a negative effect on the Cu concentration in leaf as compared to the rest of the treatments. Also, the Fe^{3+}:G7 (1:3) treatment decreased the Cu and Zn concentrations in root and leaf, respectively. The Fe:Mn molar ratio was also evaluated as an index to evaluate an adequate Fe nutrition, which should be within the optimal range 1.5-2.5 in healthy plants according to Adriano (2001). With the exception of the Fe^{3+}:G7 (1:0.5), the Fe:Mn molar ratio was within the range of 1.5-2.5 for the Fe treatments while the –Fe treatment presented a low value.

Table IV.2: Cu, Mn and Zn concentrations in tomato plants tissues and Fe/Mn ratio in leaf at 15 DAT under hydroponic conditions.

Treatment	Mn (mg Kg^{-1} DW)			Fe:Mn	Zn (mg Kg^{-1} DW)			Cu (mg Kg^{-1} DW)		
	Leaf	Stem	Root	Leaf	Leaf	Stem	Root	Leaf	Stem	Root
-Fe	49.6^{a}	10.1^{b}	29.1^{a}	0.39^{d}	40.6^{a}	21.7^{a}	29.7^{a}	6.03^{a}	3.16^{ns}	14.6^{a}
Fe^{3+}:G7 (1:0.5)	39.3^{b}	10.1^{b}	34.0^{a}	1.40^{c}	17.9^{b}	15.7^{c}	26.7^{ab}	5.99^{a}	3.19	9.22^{bc}
Fe^{3+}:G7 (1:1)	43.3^{ab}	12.2^{ab}	18.4^{b}	1.53^{bc}	17.0^{b}	19.2^{b}	15.2^{d}	7.61^{a}	3.20	8.04^{c}
Fe^{3+}:G7 (1:2)	29.6^{c}	12.9^{a}	18.9^{b}	1.84^{ab}	14.9^{bc}	15.0^{c}	19.9^{bcd}	5.81^{a}	2.51	9.63^{bc}
Fe^{3+}:G7 (1:3)	25.4^{c}	10.1^{b}	22.4^{b}	2.18^{a}	11.1^{c}	17.0^{bc}	18.2^{cd}	5.89^{a}	3.36	7.93^{c}
Fe^{3+}:HBED	29.2^{c}	9.5^{b}	20.6^{b}	1.68^{bc}	18.1^{b}	14.9^{c}	23.5^{abc}	4.13^{b}	2.53	12.3^{ab}

Different letters in the same column indicate significant differences among treatments according to Duncan´s test ($p \leq 0.05$). ns, not significant.

4.3.5 Effect of the Fe complexes on soybean seedlings under calcareous soil conditions

No significant differences among treatments were found in the plant growth, nor for the shoot and root DW (average data: 1.09 ± 0.10 and 0.28 ± 0.02 g $plant^{-1}$, respectively) or shoot and root length (average data: 45.9 ± 8.24 and 20.0 ± 1.41 cm $plant^{-1}$, respectively). The chlorophyll indices by Dualex readings were recorded for all the leaf levels during the experiment but the evolution of the third and fifth leaf levels (from 15 DAT) were used to describe the changes induced by the Fe treatments (Fig. IV.5a and IV.5b, respectively). Attending to the third leaf level, the Fe^{3+}:EDTA reached the highest chlorophyll index at 7 DAT, and no differences were presented among the other treatments with respect to the –Fe treatment at that time. At the end of the experiment, all the Fe treatments except of the Fe^{3+}:G7 (1:0.5) were significantly different from the –Fe treatments, showing a recovery in the chlorophyll index with leaf re-greening. For the fifth leaf level, Fe^{3+}:G7 (1:1, 1:2 and 1:0.5) corrected the Fe chlorosis similar to Fe^{3+}:EDTA at 21 DAT, whereas Fe^{3+}:G7 (1:3) and Fe^{3+}:G6 (1:1) along with the –Fe treatment presented the lowest values.

The highest Fe concentration in leaf was presented by the Fe^{3+}:G7 (1:2), which did not present significant differences in comparison with Fe^{3+}:EDTA at 7 DAT (Table IV.3). For the stem, the Fe^{3+}:EDTA presented the highest Fe concentration, whereas Fe^{3+}:G7 (1:2), Fe^{3+}:G6 (1:1) and the –Fe treatment presented the lowest. At 21 DAT, the Fe^{3+}:G7 (1:0.5, 1:1 and 1:2) presented the highest Fe concentration in leaf similarly to the Fe^{3+}:EDTA, but Fe^{3+}:G7 (1:3) and Fe^{3+}:G6 (1:1) presented the lowest values, similarly to the –Fe treatment at both 7 and 21 DAT. Similar Fe concentrations were found in the root for all the treatments with the exception of the Fe^{3+}:G7 (1:1) presenting the highest Fe concentration, but this data must be taken with care since they could be affected by the precipitation of Fe which may be not completely removed by washing, considering the high values obtained in all the cases. The soluble and the available Fe concentration in soil were also analyzed, but the quantification of the soluble Fe was not possible because of the instrument detection limit. The highest value in the available fraction was obtained by the Fe^{3+}:EDTA (1.8-fold) followed by the Fe^{3+}:G7 (1:1) (1.2-fold) as compared to the –Fe treatment.

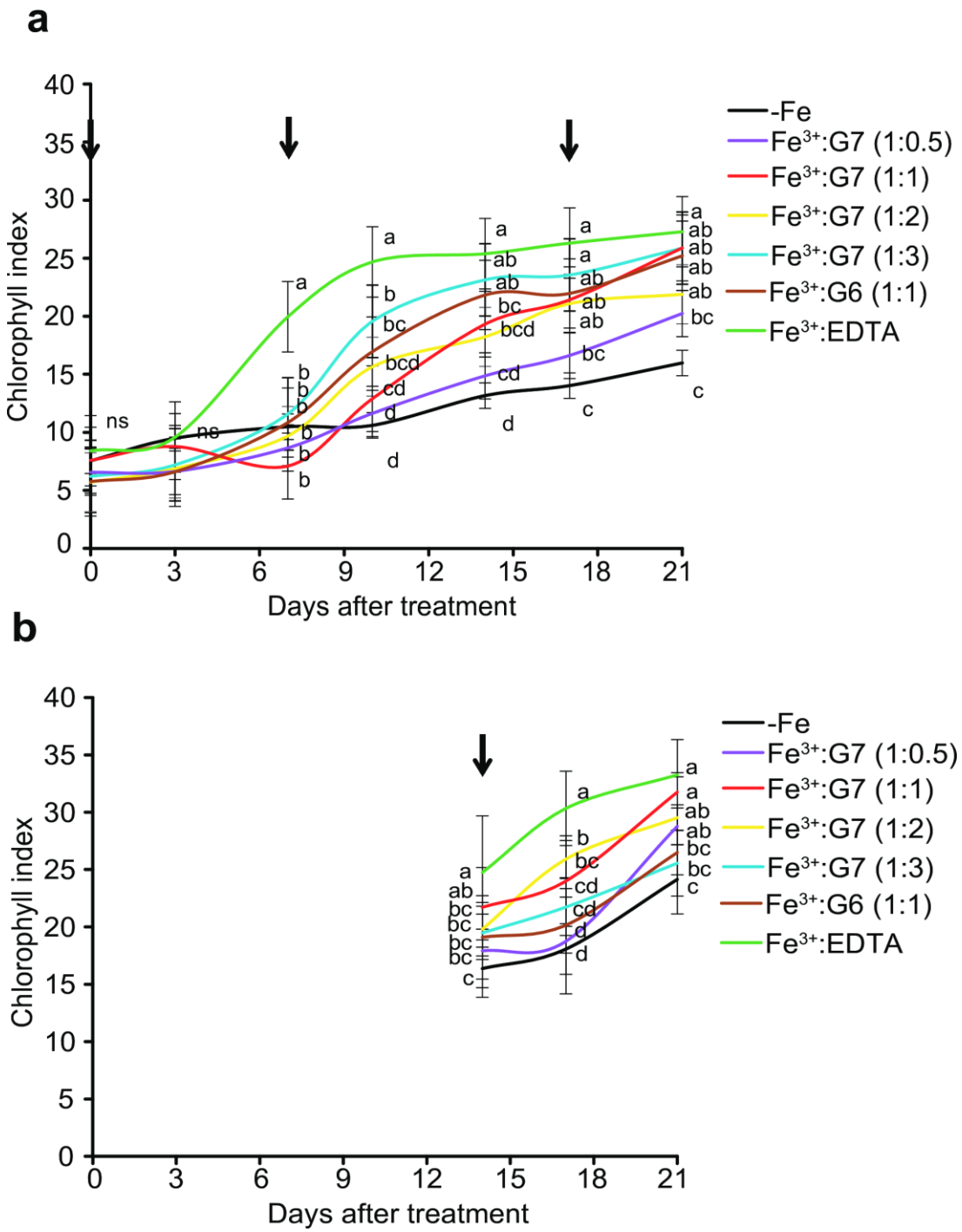

Fig. IV.5: Effect of the treatments applied on the chlorophyll index in the soybean plants grown in calcareous soil measured by Dualex in a) the third leaf level and b) fifth leaf level. Error bars indicate the standard error (N=6). Different letters represent significant differences among treatments at each day following Duncan test ($p \leq 0.05$ level). ns, not significant. Arrows indicate the days of application of the treatments.

Table IV.3: Fe concentrations in soybean tissues at 7 and 21 DAT in calcareous soil, and the available fraction of metals extracted from soils at 21 DAT.

Treatment	7 DAT (mg Kg^{-1} DW)		21 DAT (mg Kg^{-1} DW)			Available metal in soil (mg Kg^{-1})			
	Leaf	Stem	Leaf	Stem	Root	Fe	Mn	Zn	Cu
-Fe	56.1^{cd}	29.7^{d}	42.6^{c}	35.7^{a}	618^{b}	6.31^{c}	5.51^{c}	0.91^{c}	1.03^{d}
Fe^{3+}:G7 (1:0.5)	64.4^{bc}	45.8^{bc}	58.5^{a}	20.1^{b}	662^{b}	6.46^{c}	4.66^{c}	0.88^{c}	0.95^{cd}
Fe^{3+}:G7 (1:1)	66.9^{bc}	33.9^{cd}	54.1^{a}	19.5^{b}	928^{a}	14.3^{b}	15.5^{b}	3.71^{a}	2.87^{a}
Fe^{3+}:G7 (1:2)	83.4^{a}	31.8^{d}	51.5^{ab}	24.0^{b}	791^{ab}	9.20^{c}	6.92^{c}	2.10^{b}	1.71^{bc}
Fe^{3+}:G7 (1:3)	47.3^{d}	51.3^{b}	45.7^{bc}	18.9^{b}	632^{b}	5.76^{c}	6.13^{c}	0.85^{c}	0.87^{cd}
Fe^{3+}:G6 (1:1)	49.3^{d}	30.5^{d}	43.7^{c}	18.4^{b}	589^{b}	6.08^{c}	8.00^{b}	0.66^{bc}	0.97^{cd}
Fe^{3+}:EDTA	72.5^{ab}	70.6^{a}	56.4^{a}	26.9^{b}	642^{b}	22.6^{a}	22.0^{a}	2.63^{bc}	2.58^{b}

Different letters in the same column indicate significant differences among treatments according to Duncan´s test ($p \leq 0.05$). ns, not significant.

No differences among treatments were shown in the Mn concentration in leaf or stem at 7 DAT, whereas Fe^{3+}:G7 (1:1) presented the highest concentration at 21 DAT in all the soybean tissues analysed as compared to the rest of Fe^{3+}:HBED (Table IV.4). The Fe^{3+}:G7 (1:3) and Fe^{3+}:G6 (1:1) presented the lowest concentrations similar to the -Fe at 21 DAT in all the soybean tissues. Regarding to the Fe:Mn molar ratio in leaf , the highest values were obtained by the Fe^{3+}:G7 (1:2) and the Fe^{3+}:EDTA at 7 DAT while at 21 DAT they corresponded to the Fe^{3+}:G7 (1:0.5) and the Fe^{3+}:EDTA. For the Zn concentration, the highest value was presented by the Fe^{3+}:EDTA at both 7 and 21 DAT in all the soybean tissues, whereas the Fe^{3+}:G7 (1:3) and the Fe^{3+}:G6 (1:1) presented the lowest at both 7 and 21 DAT in leaf and root. The Fe^{3+}:G7 (1:1) increased the Zn concentration at 7 DAT in leaf and at 21 DAT in stem similar to the Fe^{3+}:HBED. For the Cu concentration in leaf, no differences were observed among the Fe treatments with G6 or G7 at 7 DAT, whereas both control treatments showed the lowest Cu concentration. A similar result was observed in leaf at 21 DAT for both control treatments along with Fe^{3+}:G7 (1:3) and the Fe^{3+}:G6 (1:1). With the exception of the Fe^{3+}:EDTA, these treatments also presented the lowest Cu concentration in the stem. In contrast, the Fe^{3+}:G7 (1:1) presented the highest Cu concentration at 21 DAT in all soybean tissues as compared to the Fe^{3+}:HBED. The soluble fraction of the micronutrient concentrations (Table IV.3) was restrained by the instrument detection limits, whereas the available fraction showed an increase of 1.5-2.0-fold by the Fe^{3+}:EDTA and the Fe^{3+}:G7 (1:1) for all the micronutrients as compared to the rest of the treatments.

Table IV.4: Cu, Mn and Zn concentrations in plant soybean plants tissues at 7 and 21 DAT in calcareous soil.

Treatment	Sampling (DAT)	Mn (mg Kg^{-1} DW)			Fe:Mn	Zn (mg Kg^{-1} DW)			Cu (mg Kg^{-1} DW)		
		Leaf	Stem	Root	Leaf	Leaf	Stem	Root	Leaf	Stem	Root
-Fe	7	73.5^{ns}	13.8^{ns}	---	0.76^{c}	39.0^{c}	14.0^{ns}	---	5.83^{b}	7.50^{bc}	---
Fe^{3+}:G7 (1:0.5)		72.1	14.9	---	0.95^{abc}	41.0^{c}	16.3	---	14.7^{a}	12.4^{a}	---
Fe^{3+}:G7 (1:1)		84.5	18.9	---	0.83^{bc}	55.5^{ab}	17.4	---	15.6^{a}	12.9^{a}	---
Fe^{3+}:G7 (1:2)		80.7	19.0	---	1.10^{ab}	47.0^{bc}	15.1	---	15.5^{a}	4.30^{c}	---
Fe^{3+}:G7 (1:3)		64.1	15.9	---	0.75^{c}	42.4^{c}	14.7	---	14.8^{a}	7.40^{bc}	---
Fe^{3+}:G6 (1:1)		63.6	16.2	---	0.82^{bc}	49.3^{bc}	15.4	---	16.0^{a}	10.1^{ab}	---
Fe^{3+}:EDTA		71.4	15.8	---	1.15^{a}	62.2^{a}	17.2	---	5.88^{b}	11.8^{a}	---
-Fe	21	102^{c}	14.3^{c}	19.8^{c}	0.43^{b}	42.0^{bc}	8.97^{b}	20.8^{cd}	10.9^{cd}	3.09^{c}	16.4^{b}
Fe^{3+}:G7 (1:0.5)		107^{c}	14.6^{c}	19.2^{c}	0.55^{a}	46.4^{b}	11.8^{b}	35.1^{a}	14.0^{ab}	2.96^{c}	22.6^{a}
Fe^{3+}:G7 (1:1)		161^{a}	23.0^{a}	46.6^{a}	0.35^{b}	47.1^{b}	16.4^{a}	25.3^{b}	15.0^{a}	5.48^{a}	23.0^{a}
Fe^{3+}:G7 (1:2)		128^{b}	18.8^{b}	33.9^{b}	0.43^{b}	40.9^{bc}	11.8^{b}	24.2^{bc}	13.2^{ab}	3.97^{b}	13.6^{c}
Fe^{3+}:G7 (1:3)		106^{c}	15.1^{bc}	18.5^{c}	0.43^{b}	27.2^{d}	9.10^{b}	22.9^{bcd}	10.6^{d}	2.83^{c}	17.8^{b}
Fe^{3+}:G6 (1:1)		101^{c}	15.2^{bc}	19.2^{c}	0.41^{b}	31.5^{cd}	9.81^{b}	19.5^{d}	10.4^{d}	3.23^{c}	13.6^{c}
Fe^{3+}:EDTA		89^{c}	16.3^{bc}	33.7^{b}	0.65^{a}	83.9^{a}	17.6^{a}	35.4^{a}	12.7^{bc}	4.98^{a}	16.3^{b}

Different letters in the same column indicate significant differences among treatments according to Duncan´s test ($p \leq 0.05$). ns, no significant.

4.4 Discussion

4.4.1 Complexing capacity of the gluconate and heptagluconate and characterization in function of the Fe:ligand ratios

For both G6 and G7 the MCC with Fe^{3+} was higher than that with Fe^{2+} inorganic salts, indicating a higher stability of the Fe^{3+} complexes than for the Fe^{2+} complexes at high pH. This parameter has been confirmed by the low complexed fraction by Fe^{2+}:G7 (1:2) at pH 9 mentioned above, as a consequence of the formation of Fe oxides and hydroxides and, thus, decreasing the solubility of the Fe (Carrasco *et al.*, 2012; Kovács *et al.*, 2013). The presence of a Fe^{3+} phase in the Mössbauer spectrum of the Fe^{2+}: G7 (1:2) (see Fig. IV.3b) also confirms the oxidation of these complexes. The higher complexing capacity with Fe^{3+} as compared to the Fe^{2+} salts, have been also described for other complexes such as the lignosulfonates (Carrasco *et al.*, 2012). These complexing agents present a wide variety of functional groups such as carboxylic and hydroxyl groups, which are also present in G7 and G6. The presence of other complexing agents in the complexing agents G6 and G7 used was discarded by the ^{13}C NMR and FTIR spectra (Fig. IV.2), thus confirming that the higher Fe^{3+} complexing capacity of G7 than for the G6 was not due to impurities. These results are consistent with those reported by López-Rayo *et al*. (2014), where G7 presented a better complexing capacity than G6 for Mn in solution. Also, Clemens *et al*. (1990) hypothesized that G7 has a higher complexing capacity than G6 due to previous studies that demonstrated its effectiveness to correct Fe chlorosis in calcareous soils.

The molecular weight distribution over 5000 Da for Fe^{3+}:G7 complexes at different molar ratios (Fig. IV.3f) is in agreement to the molecular weights of Fe^{3+}:sugars previously reported (Tonković *et al.*, 1983) analyzed by GFC on Sephadex® G-25 and G-100. Silva *et al.*, (2009), also observed the formation of oligomeric species for a 1:2 Fe^{3+}:citrate mixture at neutral pH. The high molecular weight obtained by the different molar ratios of the Fe^{3+}:G7 is attributed to the formation of polynuclear structures, as was confirmed by the Mössbauer spectrum of the Fe^{3+}:G7 (1:2) (see Fig. IV.1a). The formation of the polynuclear structures in the Fe^{3+}:G7 complexes involves carboxylate oxygen and deprotonated alcoholic hydroxy groups (Nagy *et al.*, 1986), as was detected in the FTIR spectra of the Fe^{3+}:G7 complexes (Fig. IV.2d). This FTIR spectra showed the binding of Fe^{3+} with carboxylic and hydroxyl groups, as well as the interaction Fe-O that can take place in the formation of the polynuclear complexes (Tonković *et al.*, 1983).

4.4.2 Effectiveness of the complexes at different Fe:ligand ratios to provide Fe under hydroponic conditions

The Fe^{3+}:G7 complexes at all the molar ratios were able to correct the Fe chlorosis when they were applied at the same Fe dose than the Fe^{3+}:HBED, based on the chlorophyll indices results (see Fig. IV.4). Moreover, the Fe concentration in the leaves did not show significant differences among the Fe treatments, being all within the Fe sufficient concentration range (50-150 µg g^{-1} DW) described by Marschner (2012). According to Ejraei (2013), Fe plays an important role in the growth and development of tomato. Therefore, the increase in the length and the DW of roots for the Fe^{3+}:G7 (1:1) and the Fe^{3+}:G7 (1:2) can be an indicator of the ability of these complexes to provide Fe in hydroponic conditions. Carrasco-Gil *et al*. (2012), also observed an increase in the DW of roots after the application of Fe to deficient tomato plants grown under hydroponic conditions. The effect of the treatments in the Fe nutrition was visible compared to the Fe-deficient plants, which presented a low chlorophyll index (see Fig. IV.4), and visible symptoms of chlorosis together with low Fe concentrations in the plant tissues. These plants also showed a marked decrease in the DW and root length, which values were similar to the Fe-deficient tomato plants grown under hydroponic conditions of other authors (Tomasi *et al*., 2009; Zamboni *et al*., 2012). The high Fe concentration in the root of plants treated with Fe^{3+}:G7 (1:05) could suggest a possible Fe precipitation on the root surface, and, as a consequence of the low percentage of Fe TR to leaf (1.2-fold) as compared to the Fe^{3+}:HBED (Table IV.1), related to its low stability. This accumulation was also observed by Kovács *et al*. (2016) when using Fe^{3+}:citrate but no when stable synthetic chelates were used. In contrast, the high percentage of Fe TR presented by the Fe^{3+}:HBED (Table IV.1) was confirmed by a 2-fold increase in the Fe concentration in stem as compared to the rest of the treatments. This result is in agreement with Martín-Fernández *et al*. (2017b), who demonstrated a high percentage of Fe TR when the Fe^{3+}:HBED was applied in early growth stages. A similar percentage of Fe TR to the Fe^{3+}:HBED was presented by the Fe^{3+}:G7 (1:1) compared to the rest of molar ratios. These differences may be due to the different stability of the complexes, as a main consequence of the different molar ratios used for their preparation. According to Carrasco *et al*. (2012), the Fe:LS molar ratio influenced in the coordination sites involved in the formation of Fe complexes.

The Mn concentration in leaves of the plants treated with the complexes were above the critical deficiency concentrations (10-20 µg g^{-1} DW) indicated by Marschner (2012), presenting the Fe^{3+}:G7 (1:1) the highest concentration in leaf and Fe^{3+}:G7 (1:2) in the

stem (Table IV.2). The larger elongation of the root for both treatments may be contributing to the better Mn uptake (Table IV.2). Because the G7 has shown to be an efficient complexing agent to maintain the Mn in solution under hydroponic conditions according to López-Rayo *et al*. (2014), it may explain the higher Mn uptake obtained in our experiment as compared to the Zn and Cu. Besides, these results suggest that a higher Mn uptake by the plant may be promoted by the application of Fe^{3+}:G7 (1:1 and 1:2) under the same conditions. In contrast, the Fe^{3+}:G7 (1:3) showed a low Mn concentration, as well as the lowest Zn concentration in leaf, even lower than the critical deficiency concentration (15-20 µg Zn g^{-1} DW) established by Marschner (2012). The low concentrations of micronutrients obtained by Fe^{3+}:G7 (1:3) can be due to the combined effect of an excess of complexing agent and a low Fe concentration. These explanation was supported by the results obtained by Wallace *et al*. (1983), where a Cu deficiency was obtained in bush bean plants grown under hydroponic conditions growth with a low Fe concentration (10^{-5} M) and an excess of EDTA. In the case of the Cu in leaf, the Fe^{3+}:G7 complexes presented concentrations above the critical deficiency concentrations (1-5 µg g^{-1} DW) in agreement with Marschner (2012), but not the Fe^{3+}:HBED treatment (Table IV.2). High concentrations of Mn, Cu and Zn in some tissues of the Fe-deficient plants were also observed in tomato grown under hydroponic conditions in Fe-free conditions (Carrasco-Gil *et al*., 2016).

4.4.3 Effectiveness of Fe:G7 and Fe:G6 complexes to provide Fe in calcareous soil conditions

This second experiment in calcareous soil was performed with soybean plants instead of tomato plants. Tomato has shown low sensitivity to Fe deficiency (Lucena *et al*., 2008) and, consequently, lower differences are expected among the Fe treatments under soil conditions. Despite the soil used was selected for its low Fe availability, this could be enough to supply sufficient Fe to the tomato plants. Moreover, the Fe^{3+}:EDTA presented the highest chlorophyll index at 7 DAT (Fig. IV.5a) and, both, the Fe^{3+}:EDTA and Fe^{3+}:G7 (1:2) presented the highest Fe concentration in leaf at 7 DAT (Table IV.3). Iron chelates have shown a faster effect for the Fe chlorosis recovery than the Fe complexes. A long-lasting effect prevails in these compounds because the Fe is mainly accumulated in the soil available fraction (Table IV.3); however, the remaining Fe in the soluble fraction was not analyzed in our experiment due to the instrumental limitations. This long-lasting effect of the studied Fe complexes such as Fe^{3+}:G6 (1:2) and Fe^{3+}:LS, and the fast effect of the Fe chelates such as Fe^{3+}:EDDHA have been also observed by Martín-Fernández *et al*. (2017a). At 21 DAT, the Fe^{3+}:G7 (1:0.5 and 1:3) and

Fe^{3+}:G6 (1:1) showed that the chlorophyll indices for the third and fifth leaf level were variable (Fig. IV.5a and IV.5b, respectively), except to the Fe^{3+}:G7 (1:1 and 1:2) that presented a similar increase to the Fe^{3+}:EDTA for both leaf levels. The fact that re-greening occurred in both leaf levels for the Fe^{3+}:G7 (1:1 and 1:2) suggests a higher Fe distribution in the leaves, similar to the Fe^{3+}:EDTA, as has been shown by Martín-Fernández *et al*. (2017a) for Fe^{3+}:G6 (1:2) and Fe^{3+}:LS. In addition, the chlorophyll indices measured in the fifth leaf level presented a similar tendency to the total Fe content in leaves. Most of them were above the Fe critical concentrations (45-50 mg kg^{-1} DW in leaf) in soybean estimated by Adams *et al*. (2000), with the exception of Fe^{3+}:G6 (1:1) and Fe^{3+}:G7 (1:3), which values were lower or closer to the critical concentration. A Fe:Mn molar ratio in leaf within the range 1.5-2.5 indicates an adequate nutrition according to Adriano (2001) but, in this study, values below 1.5 were obtained due to the low Fe dose used in the treatments to obtained a better comparison of the treatments. However, at 7 DAT, the values were higher than the values obtained by García-Marco *et al*. (2006) under hydroponic conditions, with soybean (0.67-0.54) grown for 14 days with low Fe concentrations of Fe^{3+}:EDDHA. At 21 DAT, only the Fe^{3+}:EDTA and the Fe^{3+}:G7 (1:0.5) were higher than those values since our study was conducted for 7 days more.

Fertilization with Fe^{3+}:G6 (1:1) resulted in a low chlorophyll index at 21 DAT in the fifth leaf level which can be associated to its lower complexing capacity in comparison with G7 (Fig. IV.1), which has already been hypothesized by Clemens *et al*. (1990). This treatment also showed a decrease in the concentrations of Fe, Mn, Zn and Cu in soybean tissues (Table IV.3 y IV.4). This fact is in good agreement with the low stability of G6 to maintain Mn and Zn in calcareous soil previously reported (López-Rayo *et al*., 2015).

Based on the results obtained in these experiments, the influence of the variation of the Fe:ligand ratio in the complexes may affect the following chemical mechanisms for the Fe stability and availability to the plants: (I) When the Fe^{3+}:G7 (1:3) ratio is considered, the equilibrium of the complexation reaction is shifted to the complexes formation due to the excess of ligand. As a result, the bioavailability of the other micronutrients is reduced, and even the Fe uptake by the plants can be retarded. (II) When the Fe^{3+}:G7 (1:0.5) ratio is considered, the equilibrium of the complexation reaction is shifted to the release of Fe^{3+} due to a decrease in the complexing agent. Because of the high pH in the media, the Fe is more susceptible to precipitation as Fe-oxyhydroxides on root surfaces, thus, reducing the Fe available to plants and affecting the Fe translocation. (III) When the Fe^{3+}:G7 (1:1) and Fe^{3+}:G7 (1:2) ratios are considered, the equilibrium

among micronutrients, as well as the stability of the Fe and the complexing agent seems to be promoted. This effect could be related to the affinity of the metal:ligand that can avoid competition among micronutrients and the Fe-oxyhydroxides precipitation.

The proposed chemical mechanistic behavior of the studied complexes are consistent with the low concentrations of Fe, Mn and Zn presented by soybean plants treated with Fe^{3+}:G7 (1:3) (Table IV.3 and IV.4), as well as with the results obtained for the Fe^{3+}:G7 (1:3) under hydroponic conditions (Table IV.2). This fact was also noticed by the low chlorophyll index presented in the soybean at 21 DAT (Fig. IV.5b). This decrease in the plant uptake of Fe, Mn, and Zn associated with the excess of ligands have already been described for other ligands such as DTPA, BPDS and EDTA (Lindsay and Schwab, 1982; Wallace *et al.*, 1983). In our work the Fe^{3+}:G7 (1:1) showed also the highest Mn and Cu concentrations (Table IV.4), indicating that this molar ratio can be optimal for keeping and adequate levels of these micronutrients in the plants, which was also supported by the highest Mn concentration founded by the experiment under hydroponic conditions (Table IV.2). So, although Fe^{3+}:G7 (1:2) presented a low Fe complexed fraction, it was able to sufficiently provide Fe to the plants due to the formation of polynuclear Fe complexes, that can present variable stability depending on the Fe:ligand ratio, confirming the hypothesis of this study. As it have been observed by previous studies (Stevenson, 1982; Garcia-Mina, 2006), where the complexes stability is high at low Fe:humic substances ratios at alkaline pH (e.g 1:2 compared to 1:0.5 in this work).

4.5 Conclusions

The present work provides sufficient evidences to demonstrate that Fe can be used more efficiently from Fe complexes with G7 than with G6 prepared at room temperature, providing a more stable complex and high availability for the plant, and in both cases, a long-lasting effect due to the formation of polynuclear Fe complexes. Considering the Fe source, both complexing agents G6 and G7 have a higher affinity with Fe^{3+} than with Fe^{2+}, showing a better Fe complexing capacity and stability of the Fe^{3+} complexes at high pH. The Fe^{3+}:G7 molar ratios have shown to be critical for the Fe plant uptake under hydroponic conditions and calcareous soil. An excess of ligand with respect to the Fe^{3+} reduced the micronutrients uptake in the plant by the displacement of the equilibrium reaction towards the complex formation. The Fe^{3+}:G7 1:1 and 1:2 molar ratios were those highly improving the Fe uptake while high Fe^{3+}:G7 ratios are not adequate due to the Fe-oxyhydroxides precipitation. The results obtained

also showed that a Fe^{3+}:G7 equimolar ratio can improve the uptake of other micronutrients such as Mn and Cu. Thus, the Fe^{3+}:G7 at equimolar ratio and 1:2 molar ratio can be an environmentally friendly alternative to less degradable synthetic chelates to correct Fe chlorosis in strategy I plants. Further studies with Fe^{3+}:G7 ratios of 1:1 and 1:2, but of low molecular weight synthetized according to other methodologies, as described by Nikolić *et al.* (2014) would contribute to improving the knowledge of their effectiveness and their long-lasting effect.

V. Implications of the Mn:ligand ratio in the Mn uptake by *Glycine max* plants fertilized with heptagluconate and gluconate

Resumen

El riesgo ambiental por la aplicación de quelatos sintéticos ha promovido la búsqueda de complejos biodegradables para corregir la deficiencia de Mn en plantas. En este estudio, se evaluó la capacidad para proveer Mn a las plantas por los ligandos biodegradables heptagluconato (G7) y gluconato (G6) y su relación con la relación Mn^{2+}:ligando. Por ello se realizaron ensayos biológicos en condiciones de hidroponía y suelo calizo cultivando plantas de soja y comparando con el quelato sintético EDTA. El G7 resultó una alternativa biodegradable al EDTA para proveer Mn, manteniendo un adecuado balance nutricional en comparación con el G6 que redujo la absorción de Fe por las plantas. La eficacia de los complejos de Mn depende del ligando y de la relación Mn^{2+}:ligando, siendo las relaciones 1:1 y 1:2 de Mn^{2+}:G7 las más efectivas a corto plazo. De igual manera, se observó un descenso en la absorción de micronutrientes en hoja a largo plazo, probablemente como resultado de la baja dosis aplicada de complejos. Por tanto, se debería considerar incrementar la dosis y frecuencia de aplicación de estos compuestos al suelo.

Abstract

The environmental risk of synthetic chelates application has promoted the implementation of biodegradable complexes to correct Mn-deficient plants. In this study, the biodegradable ligands of heptagluconate (G7) and gluconate (G6) were used to test the influence of the Mn^{2+}:ligand ratio in their fertilizers capacity to provide Mn to the plants. The efficacy of these complexes to correct Mn-deficient soybean was evaluated in hydroponics and calcareous soil conditions and compared to synthetic chelate EDTA. The G7 resulted in a biodegradable alternative to EDTA to supply Mn, maintaining an adequate nutritional balance as compared to the G6 that diminished the Fe uptake by the plants. The Mn complexes efficacy depended on the ligand and the Mn:ligand ratio, the 1:1 and 1:2 molar ratio of Mn^{2+}:G7 were found to be more effective in the short-term. The results also pointed out that the micronutrient uptake by plants decreased, probably caused by the complexes low dose applied. Thus, an increase in the dose and frequency of those ratios should be taken into account when are applied to the soil.

5.1 INTRODUCTION

Manganese (Mn) is a micronutrient required in low concentrations for plant growth, which is uptaken from the soil solution as Mn^{2+} (Marschner, 2012). However, certain soil conditions such as high pH in the range of 7.3-8.5 and high bicarbonate or $CaCO_3$ content promote the formation of carbonates such as $MnCO_3$ (rhodochrosite) (Norvell, 1988) lowering their solubility. The Mn availability in the soil also depends on the redox potential, so the Mn^{2+} can be easily oxidized to Mn^{3+} and Mn^{4+} in calcareous soil conditions, forming precipitates such as manganite (γ-MnOOH) and pyrolusite (β-MnO_2) (Norvell, 1988). The low availability and, thus, uptake of these compounds cause Mn deficiency in susceptible crops such as soybean, wheat, bean, oats, spinach, apple and orange (Reuter *et al*., 1988), affecting their yield and nutritional quality (Marschner, 2012).

The synthetic chelate application such as DTPA (diethylene triamine pentaacetic acid), EDTA (ethylenediaminetetraacetic acid) and HEEDTA (hydroxylethyl ethylene diamine triacetic acid) are the most widely used Mn sources in alkaline solution and calcareous soil (Boxma and de Groot, 1971; Reuter *et al*., 1988; López-Rayo *et al*., 2014, 2015). However, the persistence of these compounds in the environment along with their strong ability to complex heavy metals and to dissolve phosphates, present an environmental risk in aquatic systems promoting the search of low-cost and biodegradable complexing agents (Pinto *et al*., 2014).

Most studies with the biodegradable complexing agents of gluconate (G6) or lignosulfonate (LS) have been limited to the evaluation of multi-micronutrient formulation containing Fe, Mn and Zn to provide Mn to plants grown under hydroponics (López-Rayo *et al*., 2016) and calcareous soil conditions (López-Rayo *et al*., 2015) or macronutrient fertilizers coated with Mn-lignosulfonate (Novillo *et al*., 2001; Martín-Ortiz *et al*., 2016). These fertilizers can result in positive interactions protecting the Mn^{2+} of the oxidation, as well as in a low-cost by the additional application of macro or micronutrients (Martín-Ortiz *et al*., 2016; *Walter*, 1988). However, negative interactions due to the Mn precipitation to less soluble forms can be promoted, as a consequence of the Mn displacement by cations such as Ca^{2+}, Mg^{2+}, Zn^{2+} or Fe^{3+} (Marschner, 1988; López-Rayo *et al*., 2015).

To overcome this problem, single complexing agents have been tested in incubated calcareous soil or alkaline solution. Several studies (López-Rayo *et al*., 2012, 2014) have demonstrated the high stability of the G6 and the LS to maintain the Mn soluble at pH 9 in alkaline soil-less solutions, being similar to EDTA and HEEDTA, but a low

capacity was obtained in alkaline soil solutions (López-Rayo *et al*., 2015). *Mascagni and Cox* (1985) also demonstrated that the effectiveness of the complexes of LS to provide Mn depends on the dose applied to soybean plants grown in calcareous soil conditions, but its efficacy was similar to $MnSO_4$. Respecting the heptagluconate (G7), *López-Rayo et al.* (2014) also demonstrated that a Mn^{2+}:G7 complex was stable in alkaline soil-less solution, even at pH over 9, but in the presence of soil, a decrease of the soluble Mn after the Mn^{2+}:G7 application was observed in incubated soil solutions, being similar to the diminution observed for the $MnSO_4$ (Shaddox *et al*., 2016). Thus, the effectiveness of the Mn fertilizers strongly depends on the experimental conditions, obtaining better results in hydroponics than those in calcareous soil.

The low stability of the Mn complexes in calcareous soil conditions has been described as a result of the Mn displacement by other cations and, the complexing agent degradation by microbial activity in soil conditions (Marschner, 1988; López-Rayo *et al*., 2015). However, several studies have demonstrated that the efficacy of Mn fertilizers in calcareous soil depends on the dose applied (Mascagni and Cox, 1985; Martín-Ortiz, *et al*., 2016). In contrast, in nutrient solutions, the efficacy of the Mn complexes was related to the high metal:ligand affinity, being the competition among metals limited (López-Rayo *et al*., 2015). Regarding to the metal:ligand affinity, several studies *(*Stevenson, 1982; García-Mina, 2006), showed the effect of the metal:ligand on the complex stability and its ability to provide micronutrients to the plants. All these experiments were conducted by testing the Fe:ligand molar ratio, but not for the Mn:ligand ratio.

Then, the use of biodegradable complexing agents such as the G6 and the G7 represent another eco-friendly alternative with a low-cost (Maxwell, 2004). Besides, the high ability of G6 and G7 to complex metals at alkaline pH, especially of that of G7 (Clemens *et al*., 1990; López-Rayo *et al*., 2014) and the lower affinity of both ligands to complex Ca^{2+} in comparison to the traditional chelates (Martell and Smith, 1977; Badrinas, 2019) suggest their potential capacity as Mn fertilizers. Considering the important influence of the metal:ligand ratio preparation of the complexes, already demonstrated for the Fe complexes (Chapter IV*)*, this study explored it with two biodegradable ligands, the G6 and G7, to be used as Mn fertilizers. Firstly, the maximum complexing capacity (MCC), the fraction of complexed metal, the molecular weight distribution and the structural characterization of G7 and G6 as fertilizers of Mn were analyzed to evaluate the stability of the metals complexes. Secondly, the effectiveness of the Mn complexes at different metal:ligand ratios to supply Mn to Mn-

deficient soybean plants under hydroponic and calcareous soil conditions was evaluated.

5.2 MATERIALS AND METHODS

Pure reagents of sodium gluconate (Sigma Aldrich, ≥99%) assigned as G6, and α–sodium glucoheptonate dihydrate (G7) kindly provided by DABEER (99%, Barcelona, Spain) were used. The chelating agent ethylenediaminetetraacetic (EDTA) [Na_2EDTA, tritriplex III (Merck, 99%)] was selected for comparison. The Mn complexes of G6 and G7 were prepared with $MnSO_4 \cdot H_2O$ (PA, Panreac, 98%) of analytical grade, respectively, and type-I grade water (ISO 3696, 1987), free of organic contaminants.

5.2.1 Complexing capacity of the ligands and preparation of the Mn complexes

The maximum complexing capacity (MCC) of G6 and G7 with Mn was determined by the maximum metal that remains in solution at pH 9 after one day in the dark, following the method previously described by Villén et al. (2007).

As a result of the maximum complexing capacity with Mn of G6 and G7, the complexes were prepared based on 90% of their MCC for their characterization, resulting in approximately a Mn^{2+}:ligand molar ratio 1:1 for each complex. The complexes were prepared at pH 6-7 and then freeze-dried. The total soluble metal in the freeze-dried samples was determined, following the methods 9.2 (EU, 2003) by extraction with water, including a 0.45 µm filtration of the solution and followed by the removal of the organic compounds with H_2O_2 in acidic conditions and then, measured by atomic absorption spectroscopy (AAS, Perkin-Elmer AAnalyst 800; Shelton, CTm USA). The total complexed metal in the samples was analyzed according to EN 15962:2011 (CEN, 2011), following the same preparation than that for the soluble metal determination but rising the pH to 9 before the filtration. Also, the complexed fraction (expressed as percentage of complexed metal with respect to the soluble metal) was obtained as an index of the complex stability and to predict its effectiveness as fertilizer (Villén *et al.*, 2007; López-Rayo *et al.*, 2014).

5.2.2 Structural characterization of the Mn complexes

The structural changes of all the freeze-dried complexes after the Mn complexation were analyzed by FTIR spectra on a Bruker IFS66vd spectrometer (Germany) using KBr pellet method in the 3750-500 cm^{-1} region at a resolution of 4 cm^{-1} in the transmittance mode. The molecular weight distribution of the freeze-dried complexes was further analyzed on a glass column packed with Sephadex® G-10 (molecular

weight cut-off, MWCO >700 Da; 40-120 µm particle size distribution from Sigma Aldrich) according to the methodology described previously by *Islas-Valdez et al.* (2019). In brief, the fractions were monitored at 220 nm in a Spectrostar nano microplate reader (BMG Labtech, Ortenberg, Germany) and the Mn concentration of selected fractions analysed by AAS. The exclusion volume (V_o) was determined by blue dextran 2000 (molecular weight, MW~2000 kDa) and the total volume (V_p) with Fe^{3+}:HBED (N,N´-bis(2-hydroxyphenyl)ethylendiamine-N,N´-diacetic acid) (MW~440 Da) from 0.1 M NaCl at pH 6.

5.2.3 Plant experiments

5.2.3.1 Effectiveness of the Mn complexes in hydroponics

Soybean *(Glycine max* L., cv. RGT Speeda) seeds were grown in a growth chamber (Dycometal type CCK) with a photoperiod of 16 h to 23ºC and 40% relative humidity during the light period, and 8 h to 19ºC and 60% relative humidity during the dark period. The seeds were germinated for 10 days in perlite (1-5 mm grain; Projar, Spain) moistened with distilled water. Uniform seedlings were transferred to 4 L vessels filled with 1/5 diluted nutrient solution at pH 6 for seven days, and then, seven more days in a Mn-free full strength nutrient solution to induce the Mn deficiency. The composition of the full-strength nutrient solution (NS) was: macronutrients (mM) 1.0 Ca $(NO_3)_2{\cdot}4H_2O$, 0.9 KNO_3, 0.3 $MgSO_4{\cdot}7H_2O$, 0.1 KH_2PO_4; micronutrients (µM) 2.5 $MnSO_4{\cdot}H_2O$, 1.0 $CuSO_4{\cdot}5H_2O$, 10.0 $ZnSO_4{\cdot}7H_2O$, 1.0 $NiCl_2{\cdot}6H_2O$, 1.0 $CoSO_4{\cdot}7H_2O$, 115.5 Na_2EDTA, 35.0 NaCl, 10.0 H_3BO_3, 0.05 $Na_2MoO_4{\cdot}2H_2O$ and 5 Fe:HBED. After this period, a pair of Mn-deficient plants were transferred to 500 mL vessels containing a full-strength Mn and Na_2EDTA-free NS with a modified micronutrient concentration: 0.5 $CuSO_4{\cdot}5H_2O$, 0.5 $ZnSO_4{\cdot}7H_2O$, 0.1 $NiCl_2{\cdot}6H_2O$ and 0.1 $CoSO_4{\cdot}7H_2O$. At this time, several complexes of Mn^{2+}:G7 at different molar ratios (1:0.5, 1:1, 1:2, and 1:3) and an complex of Mn^{2+}:G6 (1:1) were prepared at pH 6-7. These treatments and the positive control Mn^{2+}:EDTA (1:1) were assayed in a concentration of 1 µM of Mn and renewed every seven days along with the NS. Besides, a Mn-free negative control (–Mn) was also included for comparison. The NS was continuously aerated and 0.1g L^{-1} of $CaCO_3$ was added to simulate calcareous soil conditions (pH 7.5). The sampling was done 14 days after the treatments application (DAT).

5.2.3.2 Effect of the Mn complexes under calcareous soil conditions.

Soybean seeds were germinated and Mn deficiency was induced as described for the hydroponic experiment. After the pre-growth period in hydroponics, three Mn-deficient

seedlings per pot were transplanted to polystyrene pots covered with aluminium foil to avoid photodegradation of Mn complexes (Hernández-Apaolaza and Lucena, 2011), filled with 180 g calcareous sand (975 g Kg^{-1} $CaCO_3$; 2-4 mm) mixed with 420 g of a clay soil (pH 8.5; 196 g Kg^{-1} sand, 396 g Kg^{-1} silt, 407 g Kg^{-1} clay; 19.4 g Kg^{-1} organic matter; 509 g Kg^{-1} total $CaCO_3$, 226 g Kg^{-1} active lime; and Soltanpour and Schwab (1977) extractable micronutrients: 8.09 mg Kg^{-1} Fe, 4.99 mg Kg^{-1} Mn, 0.46 mg Kg^{-1} Zn and 1.93 mg Kg^{-1} Cu) from Alicante (Comunidad Valenciana, Spain).

One day before transplanting, five pots per treatment were irrigated until 80% of the soil-sand mixture water holding capacity (SWHC). Three days after transplanting the Mn treatments were applied at a concentration of 4.4 µmol Mn per pot. This dose was split into three applications: 2.2 µmol on the first day, 1.1 µmol on the seventh day and 1.1 at the 14th day. In addition, a Mn-free negative control (–Mn) was assayed. During the experiment, pots were daily irrigated until 70% SWHC alternating between a 0.1g L^{-1} of $CaCO_3$/$NaHCO_3$ solution and a full-strength NS of macronutrients with 0.1g L^{-1} of $CaCO_3$/$NaHCO_3$ (pH 8-8.5). Two plant shoots per pot were sampled at 7 DAT. At 21 DAT, the remaining plant and the roots of the three plants in each pot were sampled.

5.2.4 Plant and soil analysis.

Leaf chlorophyll index was measured twice a week at different leaf levels using a portable chlorophyll meter Dualex 4 Scientific (FORCE-A, Orsay, France). Shoot length was measured after each sampling, and leaves, stems and roots were separated and washed with 0.1% non-ionic detergent (Tween 80) and 0.1 M HCl followed by tap-water and distilled water (Álvarez-Fernández *et al*., 2001). Plant tissues were dried in a forced air oven at 65 ºC for three days until constant weight to obtained the dry weight (DW) and then milled in a titanium mill (Retsch ZM200). Samples were mineralized by dry digestion in a muffle furnace at 480 ºC for 4 h followed by the acid digestion with HCl suprapur (1:1) for ash solubilization at 80 ºC per 30 min (Jones, 2001). The Fe, Mn, Cu and Zn concentrations in plant tissues were analyzed by AAS.

On completion of the experiments, the soluble and available Fe, Mn, Zn and Cu fractions in soil were determined by the extraction method proposed by Nadal *et al*. (2009) with water and DTPA solutions (Soltanpour and Schwab, 1977). The extracts obtained were acidified to eliminate the bicarbonate excess and reach a 1% acid with HNO_3 (65%, Merck) before their analysis by AAS.

5.2.5 Statistical analysis

Data were analysed by using IBM SPSS statistical software (version 23.0; SPSS Inc., Chicago, IL, USA). Differences among treatments were tested by one-way analysis of variance (ANOVA), and a Duncan post hoc test at a *p-value* < 0.05.

5.3. RESULTS

5.3.1 Complexing capacity of ligands and complexed fraction of the Mn complexes

A higher value of the MCC was obtained for G7 (0.95 mol mol^{-1} ligand) than for G6 (0.81 mol mol^{-1} ligand). As a result of these complexing capacity, both complexes were prepared at 90% of the MCC, to assure the complete complexation of Mn^{2+} (Rodríguez-Lucena *et al.*, 2010), resulting in approximately a Mn^{2+}:ligand molar ratio 1:1 for each complex.

The soluble and complexed fractions in the above mentioned freshly and freeze-dried prepared complexes were analyzed by the European official method for fertilizers (EU, 2003). For the soluble metal, the Mn^{2+}:G7 (1:1) and Mn^{2+}:G6 (1:1) complexes resulted in a 10.1 and 10.6% (g metal $100g^{-1}$ solid complex), respectively, which complies with the minimum soluble metal required of 5% for solid-state complexes (EU, 2003). Regarding the complexed fraction (CEN, 2011), the Mn^{2+}:G7 (1:1) and the Mn^{2+}:G6 (1:1) complexes presented the 97.0 and 98.4%, respectively, that also comply with the minimum complexed metal required of 80% by the regulation.

5.3.2 Structural characterization of the Mn complexes

The ligands spectra were compared to the complexes spectra to identify the involved functional groups in the Mn^{2+} complexation (Fig. V.1a-b). The bands between 3541-3157 cm^{-1}, assigned to primary and secondary hydroxyls in the ligand spectra, were hidden in the complex spectra with a strong and broadband around 3400 cm^{-1} related to the hydroxyl groups bonding water molecules (Nikolić *et al.*, 2014). The ligand spectra also show bands around 1600 and 1400 cm^{-1} assigned to the asymmetric and symmetric vibration of carboxylate anion, respectively, which were shifted to lower wavenumbers in the complexes due to the Mn coordination with the carboxylic group. The Mn coordination with secondary hydroxyl groups in the complex spectra was confirmed by a strong band around 1100 cm^{-1}, contrary to the secondary and primary hydroxyl groups at around 1090 and 1040 cm^{-1} in the ligand spectra, respectively. The Mn coordination with out-of-plane hydroxyl groups also was showed by a shift at 620

cm^{-1} in the complex spectra as compared to the ligand spectra with a band around 617 cm^{-1} (Nikolić *et al.*, 2014).

Based on the GFC results (Fig. V.1c-d), the UV-vis chromatograms of the Mn complexes showed a similar tendency for both ligands, indicating the formation of low molecular weight complexes (<700 Da), eluting after the V_o. Moreover the Mn^{2+} present in each fraction was measured by AAS, and this resulted in a broad peak (elution profile not shown) eluted after the V_o, which did not correspond to the Mn complex peak, indicating that most of the Mn^{2+} in the fractions was not associated with the complexing agent.

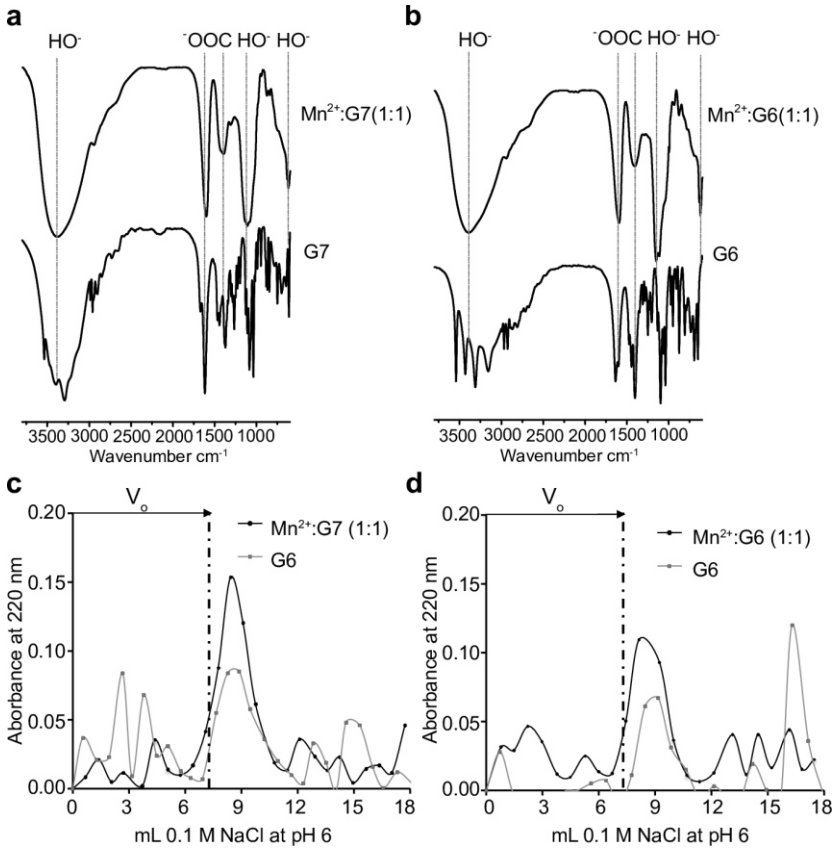

Fig. V.1: FTIR spectra of the ligands and their Mn complexes with a) heptagluconate (G7) and b) gluconate (G6). Elution profile of the absorbance measured for c) the Mn^{2+}:G7 (1:1) complex and d) the Mn^{2+}:G6 (1:1) complex passed through a Sephadex® G-10 column with 0.1 M NaCl at pH 6. The black line indicates the Mn complexes, the grey line the complexing agents. V_o indicates the exclusion volume marker in each column.

5.3.3 Mn complexes applied to soybean grown under hydroponics

All the Mn-deficient plants presented a marked decrease in the DW and length in both shoot and root as compared to the Mn treatments (Table V.1). The highest increase in

the DW and length in both shoot and root was observed by the Mn^{2+}:G7 (1:1) treatment, even higher than the Mn^{2+}:EDTA (1:1) treatment.

Table V.1: Biometric data of soybean plants at 14 DAT, grown under hydroponic conditions.

Treatment	Length (cm)		DW (g plant^{-1})	
	Shoot	Root	Shoot	Root
–Mn	42.4^{c}	42.7^{d}	1.56^{c}	0.34^{c}
Mn^{2+}:G7(1:0.5)	55.6^{b}	44.2^{cd}	2.49^{b}	0.71^{b}
Mn^{2+}:G7(1:1)	75.1^{a}	59.6^{a}	3.28^{a}	1.00^{a}
Mn^{2+}:G7(1:2)	61.7^{b}	52.0^{b}	2.74^{b}	0.83^{ab}
Mn^{2+}:G7(1:3)	62.2^{b}	49.7^{bc}	2.77^{b}	0.80^{ab}
Mn^{2+}:G6(1:1)	70.5^{a}	51.2^{b}	2.96^{ab}	0.75^{b}
Mn^{2+}:EDTA(1:1)	60.1^{b}	49.7^{bc}	2.44^{b}	0.73^{b}

Different letters in the same column represent significant differences among treatments following Duncan test ($p \leq 0.05$ level). ns, not significant

The Mn-deficient plants suffered a detriment of the chlorophyll index during the experiment (chlorophyll index of the third leaf level measured by Dualex is shown in Fig. V.2a), showing visible symptoms of interveinal chlorosis. In contrast, all the Mn treatments showed a leaf re-greening from 7 DAT. The Mn^{2+}:G7 (1:2) treatment presented a 13% increase as compared to the Mn^{2+}:EDTA (1:1) at the end of the experiment, while the Mn^{2+}:G7 (1:1) was significantly similar to both treatments.

All the Mn treatments induced an increase in the Mn content in all plant tissues as compared to the Mn-deficient soybean plants (Fig. V.2b). Within the assayed complexes, the highest Mn contents in all plant tissues were those obtained by the Mn^{2+}:G7 (1:2 and 1:1) with increases in leaf (9.70% and 6.76%, respectively), stem (34% and 18%, respectively) and root (39% and 18%, respectively) in comparison to the Mn^{2+}:EDTA (1:1) treatment. Although, the rest of the molar ratios of Mn^{2+}:G7 and the Mn^{2+}:G6 (1:1) provided similar Mn contents than the Mn^{2+}:EDTA (1:1) for all plant tissues, the Mn^{2+}:G7 (1:0.5) presented a 17% lower Mn content than the Mn:G7 (1:2 and 1:1) complexes in the leaf. For the stem, the Mn^{2+}:G7 (1:3) and the Mn^{2+}:EDTA (1:1) treatments presented a decrease in the Mn content (27% and 25%, respectively) as compared to the Mn^{2+}:G7 (1:2). The Mn^{2+}:G7 (1:3) also showed a 37% lower Mn content than the Mn^{2+}:G7 (1:2 and 1:1) for the root.

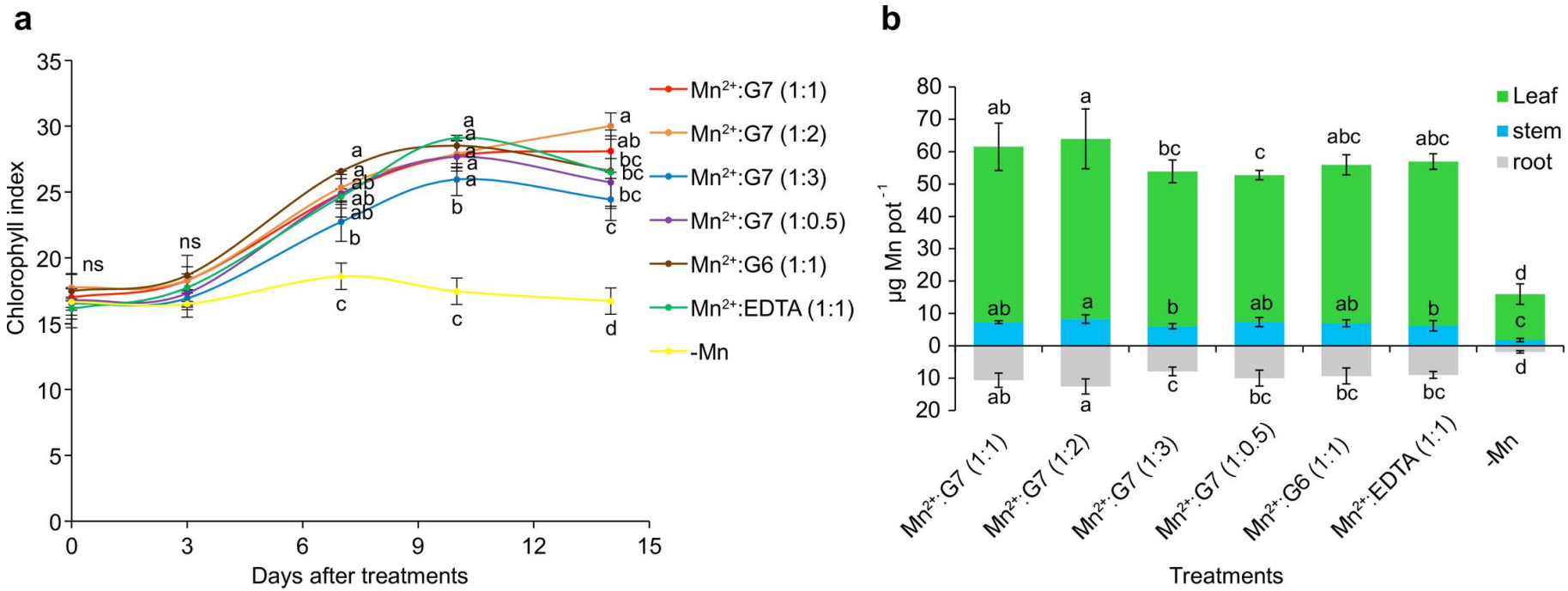

Fig. V.2: Effect of the treatments on a) the chlorophyll index in the soybean plants grown in hydroponics measured by Dualex in the third leaf level and b) the Mn content in plant tissues. Error bars indicate the standard error (N=5). Different letters represent significant differences among treatments at each day following Duncan test ($p \leq 0.05$ level). ns, not significant.

Table V.2: Fe, Zn and Cu content in plant tissues and micronutrient molar ratios in leaf at 14 DAT of soybean grown under hydroponic conditions.

Treatment	Fe (µg pot^{-1})			Zn (µg pot^{-1})			Cu (µg pot^{-1})			Fe:Mn	Zn:Mn	Cu:Mn
	Leaf	Stem	Root	Leaf	Stem	Root	Leaf	Stem	Root	Leaf	Leaf	Leaf
-Mn	79.1^{c}	14.4^{ns}	32.3^{b}	33.3^{c}	11.6^{b}	5.43^{b}	4.26^{b}	1.74^{c}	6.48^{c}	4.98^{a}	2.01^{a}	0.28^{a}
Mn^{2+}:G7(1:0.5)	100^{b}	24.7	102^{a}	40.8^{bc}	16.2^{a}	15.4^{a}	6.79^{a}	2.88^{ab}	11.5^{b}	2.10^{bc}	0.72^{b}	0.12^{bc}
Mn^{2+}:G7(1:1)	100^{b}	21.5	97.0^{a}	52.1^{a}	15.6^{a}	13.1^{a}	6.33^{a}	3.38^{a}	11.7^{b}	1.82^{c}	0.81^{b}	0.10^{bcd}
Mn^{2+}:G7(1:2)	130^{a}	20.1	78.2^{a}	49.2^{ab}	15.8^{a}	16.4^{a}	6.37^{a}	2.94^{a}	16.9^{a}	2.48^{b}	0.71^{b}	0.09^{cd}
Mn^{2+}:G7(1:3)	101^{b}	16.8	94.8^{a}	44.1^{ab}	11.4^{b}	14.6^{a}	7.62^{a}	3.03^{a}	12.2^{b}	2.08^{bc}	0.78^{b}	0.14^{b}
Mn^{2+}:G6(1:1)	98.0^{bc}	17.3	98.0^{a}	47.7^{ab}	16.3^{a}	14.1^{a}	7.06^{a}	3.47^{a}	10.7^{b}	1.98^{bc}	0.78^{b}	0.13^{bc}
Mn^{2+}:EDTA(1:1)	98.1^{bc}	17.8	67.9^{a}	42.7^{abc}	13.4^{ab}	11.7^{a}	4.79^{b}	2.29^{bc}	9.04^{bc}	1.88^{c}	0.73^{b}	0.08^{d}

Different letters in the same column represent significant differences among treatments following Duncan test ($p \leq 0.05$ level). ns, not significant.

The impact of the treatments in the other micronutrients content was also studied (Table V.2). The –Mn treatment presented the lowest values for Fe, Zn, and Cu in most of the plant tissues as compared to the Mn treatments. In contrast, the Mn^{2+}:G7 (1:2) treatment presented the highest leaf Fe content, increasing a 32% from that of Mn^{2+}:EDTA (1:1). The Fe:Mn molar ratio was also evaluated as an adequate Mn nutrition index, presenting values within the optimal range 1.5-2.5 established by Adriano (2001) in the plants supplied with Mn, whereas the –Mn treatment presented a value above 2.5. No differences were shown among the plants treated with complexes or the Mn^{2+}:EDTA (1:1) in the leaf Zn content, but all the Mn treatments, even the Mn^{2+}:EDTA (1:1) presented a lower Zn:Mn molar ratio than the –Mn treatment. The plants treated with the Mn complexes presented a higher leaf Cu content than the control –Mn treatment and the Mn^{2+}:EDTA (1:1), but the –Mn treatment showed the highest Cu:Mn molar ratio compared to the plants supplied with Mn^{2+}.

5.3.4 Efficacy of the Mn complexes in soybean grown in calcareous soil conditions

No significant differences were found among treatments in the plant growth nor for the shoot DW (average data: 1.46 ± 0.09 g per plant) or shoot length (average data: 87.0 ± 6.1 cm). The trends for the chlorophyll index was recorded for all the leaf levels during the experiment, but only the percentage increase of the third leaf level was evaluated [$\Delta Dualex=((Dualex_{7/21DAT}-Dualex_{0DAT})/Dualex_{0DAT}) \times 100$], since it was the developing leaves at the moment of the first treatment application. No differences were observed among the treatments at 7 and 21 DAT (Table V.3). However, a marked decrease in the chlorophyll index from 7 to 21 DAT was showed by the Mn^{2+}:G6 (1:1) treatment as compared to the rest of the Mn treatments, that presented increases.

Concerning the Mn content in the plant tissues (Table V.3), no significant differences were found among treatments in the stem Mn content at 7 DAT. In leaves, the plants treated with Mn^{2+}:G7 (1:2 y 1:1) and Mn^{2+}:G6 (1:1) presented a higher Mn content (45, 44 and 38%, respectively) than the Mn-deficient plants, and similar to the Mn^{2+}:EDTA (1:1) treatment. In contrast, the other molar ratios of Mn^{2+}:G7 (1:0.5 and 1:3) treatments presented a leaf Mn content similar to the –Mn treatment. At 21 DAT, no differences among treatments were found in the Mn content of any plant tissues, which values were, in any case, higher than those obtained at 7 DAT. The soluble and the available Mn concentration in the remaining soil were also analyzed, but the soluble Mn was below the detection limit in the extracts. No differences among treatments were found in the soil available Mn concentration.

Table V.3: Mn content in soybean tissues at 7 and 21 DAT in calcareous soil, and available Mn fraction extracted from soils at 21 DAT.

Treatment	7 DAT			21 DAT				Available metal in soil ($\mu g\ g^{-1}$)
	Δ Dualex	Mn ($\mu g\ pot^{-1}$)		Δ Dualex	Mn ($\mu g\ pot^{-1}$)			
	3rd level	Leaf	Stem	3rd level	Leaf	Stem	Root	Mn
–Mn	32.7^{ns}	39.7^{c}	8.66^{ns}	51.7^{ns}	94.2^{ns}	16.1^{ns}	51.3^{ns}	11.4^{ns}
Mn^{2+}:G7 (1:0.5)	31.3	44.6^{bc}	8.30	44.4	104	14.7	55.4	11.3
Mn^{2+}:G7 (1:1)	26.4	57.3^{ab}	10.4	39.7	101	19.1	42.0	11.2
Mn^{2+}:G7 (1:2)	46.3	57.7^{ab}	9.76	41.3	98.0	14.2	51.7	11.3
Mn^{2+}:G7 (1:3)	45.0	52.2^{abc}	9.53	44.4	107	16.8	63.4	10.6
Mn^{2+}:G6 (1:1)	36.4	54.9^{ab}	9.37	28.6	110	17.4	41.1	11.6
Mn^{2+}:EDTA	44.3	61.2^{a}	8.38	48.9	124	15.3	57.6	12.0

Different letters in the same column represent significant differences among treatments at each day following Duncan test ($p \leq 0.05$ level). ns, not significant.

Regarding the effect on other micronutrients, no differences among treatments in the Fe or Cu contents in the stem at 7 DAT were observed (Table V.4). Similar values were observed in the leaf Fe content of the plants with the exception of Mn^{2+}:G6 (1:1) and Mn^{2+}:G7 (1:0.5) that presented lower values than the –Mn control. All the Mn treatments induced a diminution of the Fe:Mn ratio as compared to that of the –Mn treatment. Only the Mn^{2+}:G7 (1:2) presented values similar to the –Mn treatment. The plants treated with Mn^{2+}:G7 (1:1) induced a decrease of the Zn and Cu contents in leaf, similar to that of the Mn^{2+}:EDTA (1:1), while the other Mn^{2+}:G7 molar ratios and the Mn^{2+}:G6 presented higher values, similar o even higher than the –Mn control.

At 21 DAT, no differences among treatments in the Fe content in leaf or root were observed; consequently, the Fe:Mn molar ratio did not show differences among the treatments as well. The Zn and the Cu contents in leaf were lower at 21 DAT than at 7 DAT for all the treatments. Besides, most of the Mn^{2+}:G7 treatments presented a leaf Zn content lower than that of the –Mn control, while the Mn^{2+}:G6 was similar. No differences in the leaf Cu content were observed among treatments. For the stem Fe content, no differences were observed among the treatments with complexes and the control treatments, The highest Zn and Cu content in stem was presented by the Mn^{2+}:G7 (1:2 and 1:0.5) treatments along with the Mn^{2+}:EDTA (1:1) treatment.

At the end of the experiment, the concentration of the soluble and available soil extracts were also analysed for other micronutrients. With the exception of the Mn^{2+}:G7 (1:2) treatment (Fe concentration: 0.20 ± 0.02 μg g^{-1} soil), the soluble Fe concentration in the soil for the rest of the treatments was below the detection limit of the instrumentation. Regarding the concentration of the available Fe in soil (Table V.5), the pots treated with Mn^{2+}:G7 (1:3) and Mn^{2+}:G6 (1:1) presented a decrease as compared to the –Mn control, and the rest of the Mn:G7 presented similar concentrations than the Mn^{2+}:EDTA. No differences among treatments were found in the soluble (average value: 0.030 ± 0.002 μg g^{-1} soil) or the available Zn concentrations in soil (Table V.5). All the Mn complexes presented soluble Cu concentration values around 0.019 ± 0.004 μg g^{-1} soil, similar to the –Mn control (0.020 ± 0.006 μg g^{-1} soil), while a higher value was showed by the Mn^{2+}:EDTA (1:1) treatment (0.036 ± 0.007 μg g^{-1} soil). However, the Mn^{2+}:EDTA (1:1) induced the lowest soil available Cu concentration (Table V.5), similar to the Mn^{2+}:G7 (1:3). Lower values were obtained for the other Mn^{2+}:G7 molar ratios and the Mn^{2+}:G6, presenting the Mn^{2+}:G7 (1:1) and the Mn^{2+}:EDTA (1:1) the lowest values.

Table V.4: Fe, Zn and Cu contents in soybean tissues, and Fe:Mn ratio in leaf at 7 and 21 DAT in the experiment conducted in calcareous soil.

Treatment	Sampling (DAT)	Fe (µg pot^{-1})			Zn (µg pot^{-1})			Cu (µg pot^{-1})			Fe:Mn
		Leaf	Stem	Root	Leaf	Stem	Root	Leaf	Stem	Root	Leaf
-Mn	7	40.1^{a}	22.0^{ns}	---	21.4^{b}	8.29^{a}	---	6.77^{ab}	4.88^{ns}	---	0.94^{a}
Mn^{2+}:G7 (1:0.5)		21.4^{bc}	18.4	---	26.5^{ab}	7.62^{ab}	---	8.49^{a}	4.26	---	0.48^{cd}
Mn^{2+}:G7 (1:1)		33.6^{ab}	20.1	---	11.0^{c}	4.60^{c}	---	5.10^{bc}	2.53	---	0.58^{bc}
Mn^{2+}:G7 (1:2)		43.1^{a}	17.1	---	20.5^{b}	6.07^{abc}	---	8.09^{ab}	2.75	---	0.75^{ab}
Mn^{2+}:G7 (1:3)		35.1^{ab}	16.5	---	19.4^{b}	7.51^{ab}	---	7.76^{ab}	3.82	---	0.65^{bc}
Mn^{2+}:G6 (1:1)		17.7^{c}	20.0	---	29.6^{a}	7.64^{ab}	---	8.85^{a}	3.76	---	0.32^{d}
Mn^{2+}:EDTA (1:1)		30.5^{abc}	23.3	---	10.5^{c}	5.07^{bc}	---	3.66^{c}	2.77	---	0.49^{cd}
-Mn	21	40.6^{ns}	16.4^{ab}	1384^{ns}	21.5^{a}	6.18^{b}	16.6^{a}	2.43^{ns}	2.52^{c}	4.19^{d}	0.31^{ns}
Mn^{2+}:G7 (1:0.5)		33.1	13.1^{b}	1618	15.5^{bc}	8.18^{a}	18.1^{a}	3.73	5.76^{a}	7.29^{b}	0.31
Mn^{2+}:G7 (1:1)		33.4	10.5^{b}	1127	7.15^{d}	4.00^{c}	8.63^{b}	2.99	2.95^{c}	5.69^{c}	0.32
Mn^{2+}:G7 (1:2)		28.1	15.3^{b}	1192	10.9^{cd}	8.24^{a}	12.9^{ab}	3.06	5.54^{a}	5.72^{c}	0.24
Mn^{2+}:G7 (1:3)		32.9	13.8^{b}	1124	11.4^{bcd}	5.23^{bc}	14.8^{a}	3.28	3.05^{c}	6.06^{bc}	0.31
Mn^{2+}:G6 (1:1)		26.4	22.5^{a}	1015	17.4^{ab}	5.80^{bc}	18.3^{a}	4.50	3.99^{b}	12.5^{a}	0.27
Mn^{2+}:EDTA (1:1)		29.1	16.6^{ab}	1258	9.10^{d}	8.92^{a}	14.2^{ab}	3.04	5.94^{a}	3.77^{d}	0.23

Different letters in the same column represent significant differences among treatments at each day following Duncan test ($p \leq 0.05$ level). ns, not significant.

Table V.5: The available fraction of metals extracted from soils at 21 DAT.

Treatment	Available metal in soil (µg g^{-1})		
	Fe	Zn	Cu
-Mn	7.83^{a}	0.37^{ns}	1.15^{cd}
Mn^{2+}:G7 (1:0.5)	6.46^{ab}	0.26	1.71^{a}
Mn^{2+}:G7 (1:1)	6.57^{ab}	0.27	0.92^{d}
Mn^{2+}:G7 (1:2)	6.59^{ab}	0.45	1.20^{bcd}
Mn^{2+}:G7 (1:3)	6.34^{b}	0.22	1.60^{ab}
Mn^{2+}:G6 (1:1)	4.94^{c}	0.34	1.36^{abc}
Mn^{2+}:EDTA (1:1)	6.62^{ab}	0.38	0.90^{d}

Different letters in the same column represent significant differences among treatments following Duncan test ($p \leq 0.05$ level). ns, not significant.

5.4. DISCUSSION

5.4.1 Stability of the Mn complexes at alkaline pH

The Mn MCC of G7 was higher than that of G6, which corroborates the higher complexing capacity of G7 at alkaline pH previously obtained for Fe in the Chapter IV and by Clemens *et al.* (1990). Nevertheless, the fertilizers based on complexes also should contain a minimum of 80% of complexed metal according to the European Regulation (EU, 2003). This index represents a prediction of the complex stability and thus, its efficacy to supply the micronutrients to plants. Since the Mn complexes prepared in our lab conditions complied with this requirement, it would be expected that both ligands were efficient as fertilizers of Mn under calcareous soil conditions. These results are consistent with previous studies (López-Rayo *et al.*, 2012, 2014), where a G7 and G6 presented higher stability than the EDTA to maintain the Mn soluble in alkaline solutions at pH 9, even over pH 9, the G7 showed higher capacity than the G6.

According to the chemical characterization, the Mn complexation was by coordination to the carboxylate and the secondary hydroxyl groups, as well as to the water molecules (Fig. V.1a-b). Similar conclusions were reported by previous studies of Mn^{2+}:G6 complexes (Yamaguchi and Sawyer, 1985; van Duin *et al.*, 1989; Gyurcsik and Nagy, 2000), where the stability of these complexes in alkaline solutions is attributed to the Mn coordination to the carboxyl and the hydroxyl group at the α position of these ligands, forming a five-membered ring.

The results obtained by GFC are consistent with previous studies (Bodini *et al.*, 1976; Rudnik, 2015) that suggested that at acid pH, weak Mn complexes are formed, contrary to those at alkaline pH, which are more stable. This fact is consistent with the results obtained by *Rudnik* (2015), who demonstrated that the Mn stability constants of Mn were higher with Cl^- than gluconate in acid media. In our study, 0.1 M NaCl at pH 6 was used as mobile phase to counter the interaction between the complex and the stationary phase, influencing in the Mn distribution obtained.

5.4.2 Mn plant uptake from hydroponics

The Mn-deficient plants presented a decrease in the growth, chlorophyll index and the Mn content in all the plant tissues. Similar symptoms in Mn-deficient plants grown under hydroponic conditions have been described elsewhere (Lombnæs and Singh, 2003; Carrasco-Gil *et al.*, 2016). These symptoms are the outcome of damages in the thylakoid structure and photosystem II promoting the chlorophyll degradation (Simpson and Robinson, 1984) caused by the leaf Mn concentration below the sufficiency range (20-100 mg kg^{-1} DW, Jones (2012)). Although all the Mn treatments increased the leaf Mn concentration above this sufficiency range, only the Mn^{2+}:G7 (1:1 and 1:2) treatments increased the Mn content in all the plant tissues in comparison to the rest of the treatments (Fig. V.2b). Consequently, these Mn^{2+}:G7 treatments presented high values for the chlorophyll index (Fig. V.2a) resulting from the increase of the whole-plant Mn content. The highest root elongation presented by the Mn^{2+}:G7 (1:1) treatment also may indicate its efficacy as Mn fertilizer, since the root growth is related to the Mn supply (Marschner, 2012). The ability of these molar ratios of the Mn^{2+}:G7 to provide Mn in hydroponics may be related to its metal:ligand affinity, which promotes an adequate equilibrium between the metallic micronutrients (Table V.2), limiting the Mn displacement. As have been supported in a previous experiment (Chapter IV), when Fe was applied as Fe^{3+}:G7 in the same molar ratios (1:1 and 1:2) in Fe-deficient plants under hydroponic conditions, a higher elongation of root and Mn uptake was promoted. López-Rayo *et al.* (2016) found positive correlations between the leaf Mn content and the chlorophyll index, and between the leaf Mn content and the root DW in hydroponics.

The lower capacity of the 1:3 and 1:0.5 ratios of Mn^{2+}:G7 compared to the 1:1 and 1:2 ratios to provide Mn^{2+} is attributed to the postulated mechanism for these complexes with Fe (Chapter IV). Therefore, the complexes prepared with these ratios can reduce the Mn uptake by the precipitation (1:0.5 complex) or competition (1:3 complex) between the ligand and the root for the Mn^{2+}. In the case of the equimolar complexes of

G6 and G7 with Mn^{2+}, both complexes present similar functional groups, spectra and complexed fraction, but Mn^{2+}:G6 showed a different capacity to correct the Mn deficiency in plants. This effect may be attributed to the differences in the complex structures caused by the isomerism of each ligand, being the G7 referred to the α-isomer. Since the Mn complexes form five-membered rings, the participation of the primary hydroxyl in the Mn^{2+}:G7 (1:1) complex may also be suggested, whereas the Mn^{2+}:G6 (1:1) conformation restricts this bond, explaining its low stability. A similar structure to the Mn^{2+}:G7 (1:1) is also proposed for the Mn^{2+}:G7 (1:2), preventing the formation of oxidized Mn species and maintaining the Mn soluble in alkaline solution for the plant uptake. The tentative structures for the complexes formed under our lab conditions are shown in Fig. V.3.

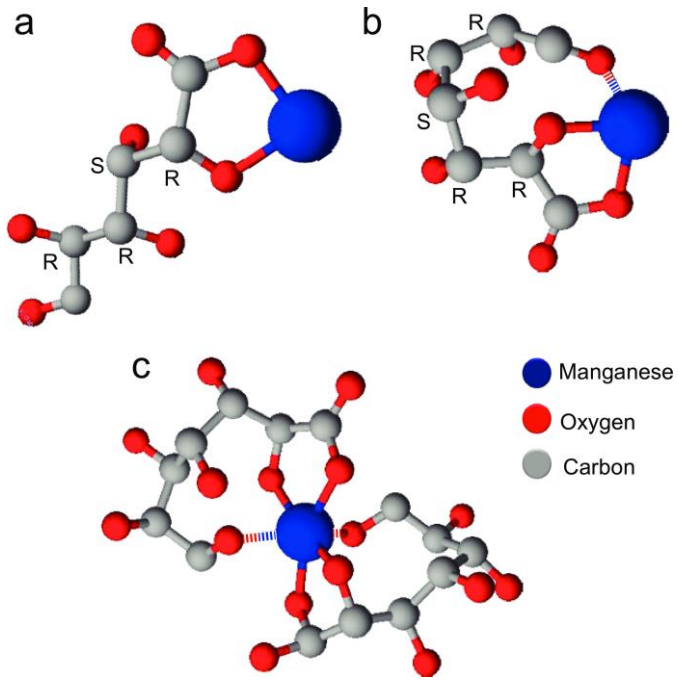

Fig. V.3 The optimal geometry of the a) Mn^{2+}:G6 (1:1), b) Mn^{2+}:G7 (1:1) and c) Mn^{2+}:G7 (1:2) complexes were obtained by using ACD/ChemSketch software program. Hydrogen and water molecules are not included, for simplicity.

Although all the treatments presented a leaf Fe concentration within the sufficiency range (50-350 mg kg^{-1} DW; Jones, 2012), the high leaf Fe content (32% higher than the –Mn control) in the plants treated with Mn^{2+}:G7 (1:2) in comparison to the rest of treatments, suggests that this complex promotes the Fe mobilization from the calcareous soil. In a previous work (Chapter IV), an increase in the short-term Fe uptake, when Fe-deficient soybean plants were fertilized with Fe^{3+}:G7 (1:2) was demonstrated. Also an increase in long-term Mn uptake was noticed, indicating that a shuttle effect may be occurring when this molar ratio is applied. Thus, when the roots

takes up Mn^{2+}, the G7 can dissolve Fe from the soil, but also the G7 can complex more Mn^{2+}. The Fe:Mn molar ratio in leaf within the range 1.5-2.5 indicated an equilibrated nutrition for all the Mn treatments, according to Adriano (2001). The value above 2.5 presented by the –Mn treatment is consistent with the values obtained by previous studies (Somers *et al.*, 1942; López-Rayo *et al.*, 2016) for Mn-deficient soybean plants. The –Mn treatment also presented the highest Zn:Mn and Cu:Mn molar ratios as compared to the Mn treatments, whose plants presented low values for both molar ratios. This result is in agreement with Kobraee and Shamsi (2015), who reported that Mn application reduced the Zn:Mn ratio for soybean shoots grown in calcareous soil. Nevertheless, all the treatments presented a leaf Zn concentration within the sufficiency range (20-50 mg kg^{-1} DW; Jones, 2012). In contrast, all treatments showed a leaf Cu concentration below the sufficiency range (10-30 mg kg^{-1} DW; Jones, 2012). The low Cu concentration in the plant tissues is caused by our washing method, which is adequate taking into account solely the micronutrient uptake and eliminating the possible contamination in the surface of the plant tissues (Álvarez-Fernández *et al.*, 2001). Thus, this concentration might be considered low rather than deficient. Low contents of Fe, Zn and Cu in most of the analyzed tissues of the Mn-deficient plants accounts for the decrease in the plant growth, despite no significant differences in their concentrations were obtained for most of the Mn treatments. These results are comparable to those obtained in Mn-deficient plants of barley and oat grown in hydroponics by Lombnæs and Singh (2003), who suggested that the antagonistic relationships among Mn with Fe, Zn and Cu depend on the plant growth and the crop, especially the antagonism between Mn and Fe. Heenan and Campbell (1983) also demonstrated that the Fe uptake in soybean grown in hydroponics was independent of the Mn concentration, and only was dependent on the Fe chelated applied. In our experiment the same Fe chelate (Fe:HBED) was applied for all the plants.

5.4.3 Responses of soybean to low doses of Mn complexes in calcareous soil

No differences among treatments were observed by the percentage increase of the chlorophyll index at any sampling time. According to *Marschner* (2012), only under severe Mn deficiency conditions, a decrease in the leaf chlorophyll is observed. In our study in calcareous soil, all treatments, including the –Mn control, presented a leaf Mn concentration within the sufficiency range (Jones, 2012). Although the soil used was selected by its low Mn availability, that could be enough to supply Mn to the plants from the rhizosphere acidification by Mn-deficient soybean plants, as has been suggested by other authors (Marschner, 1988; Gollany and Schumacher, 1993; Martín-Ortiz *et al.*,

2016). In contrast, when the Mn is completely removed in hydroponics, the chlorophyll index revealed significant differences (Fig. V.2a). This result is in agreement with López-Rayo *et al.* (2015), who demonstrated a correlation between the chlorophyll index and the Fe concentration in leaf but not with the Mn concentration under calcareous soil. It is also consistent with the decrease of the chlorophyll index between the 7 DAT and 21 DAT presented by the Mn^{2+}:G6 (1:1) treatment in this study, as a consequence of the low leaf Fe concentration at both samplings and the diminution in the available Fe in the soil. The negative effect of the Mn:G6 (1:1) treatment on the Fe uptake observed in our experiment can be associated with the low stability of G6 to maintain the Fe soluble under calcareous soil conditions demonstrated in the Chapter IV and by previous authors (Clemens *et al*., 1990; Goos and Germain, 2001).

Although the leaf Mn concentration at 7 DAT in all the treatments was within the sufficiency range, only the Mn^{2+}:G7 (1:1 and 1:2) complexes, and the Mn^{2+}:G6 (1:1) treatments showed a fast effect to provide Mn to the plants, similar to the Mn^{2+}:EDTA (1:1). Despite the Mn^{2+}:G6 (1:1) treatment increased the leaf Mn content, its application to calcareous soil should be limited due to the reasons above mentioned and a leaf Fe:Mn ratio less than 0.4, being necessary to modify the application of the other micronutrients to keep an adequate micronutrient balance. In contrast, the values of the leaf Fe:Mn ratio of the Mn^{2+}:G7 (1:1 and 1:2) along with the Mn^{2+}:EDTA (1:1) were higher to those obtained by Ghasemi-Fasaei *et al.* (2002), who reported that a Fe:Mn ratio less than 0.4 for soybean shoots grown in calcareous soil conditions is an index of imbalance between these micronutrients. The fruitful effectiveness of the Mn^{2+}:G7 (1:1 and 1:2) complexes supplying Mn to soybean plants has been related to the metal:ligand affinity that contributes to the stability of the complexes and the equilibrium among micronutrients (Chapter IV) and the tentative proposed structures (Fig. V.3). According to Humphries *et al.* (2007), the Mn^{2+} uptake is faster as free cation than in its form complexed or chelated, thus, the low molecular weight complexes formed (Fig. V.1) can promote a fast effect to supply Mn at 7 DAT similar to the Mn^{2+}:EDTA (1:1) treatment.

Neither the Mn content in any plant tissue nor the available Mn in the soil showed differences among the treatments at 21 DAT. However, the Mn^{2+}:G7 (1:1 and 1:2) complexes showed a higher effectiveness than the Mn^{2+}:EDTA (1:1) treatment to provide Mn in hydroponics (Fig. V.2a) and their efficacy to provide Mn at 7 DAT in calcareous soil conditions, suggesting that the long-term effect of the Mn complexes in soil conditions is limited by its low dose applied. This is consistent with the decrease in the leaf Zn, Cu, and Fe concentration in all the Mn treatments in the long-term, whose

values were below the sufficiency range (Jones, 2012). Consequently, all treatments presented Fe:Mn molar ratios less than 0.4 (Table V.2), indicating an imbalance between these micronutrients caused by the Fe-deficiency in leaves *(*Ghasemi-Fasaei *et al.,* 2002). This fact explains the increase in the Mn content in the long-term as an outcome of the antagonistic effect between Fe and Mn under soil conditions (Marschner, 1988), but may also a result of the rhizosphere acidification capacity of soybean (Marschner, 1988; Gollany and Schumacher, 1993; Martín-Ortiz, 2016). Although the Zn and Cu concentrations in leaf were below the range sufficiency at 21 DAT, the increase in the stem Zn and Cu content for the Mn^{2+}:G7 (1:2) treatment may also be an outcome of a shuttle effect of this complex, but to a lesser extent as outcome of the low dose applied. These results suggest that a higher dose of the Mn^{2+}:G7 (1:1 and 1:2) treatments would contribute to increase their effectiveness in long-term experiments and to provide an effective biodegradable alternative to the EDTA. In our previous work with Fe treatments (Chapter IV), the Fe^{3+}:G7 (1:1 and 1:2) complexes improved the Mn, Fe, Cu and a lesser extent the Zn uptake in calcareous soil, as a consequence of the higher ligand concentration used, as compared with this study. An increase in the dose of lignosulfonates and biodegradables Mn chelates have also suggested to improve the efficacy of Mn fertilizers under calcareous soil conditions in long-term for other authors (Mascagni and Cox, 1985; López-Rayo *et al*., 2015).

5.5 Conclusions

The results of the present study demonstrate that Mn can be better complexed by G7 than by G6, forming in both cases low molecular weight compounds. In these compounds, the Mn may be coordinated to five-membered ring for both ligands, whereas the probably binding to primary hydroxyl group in the Mn^{2+}:G7 (1:1) may improve its stability as compared to the Mn^{2+}:G6 (1:1). The 1:1 and 1:2 molar ratios of Mn^{2+}:G7 were the most effective Mn sources under hydroponic conditions and can be biodegradable alternatives to the Mn^{2+}:EDTA (1:1). These complexes also showed to be an adequate alternative in calcareous soil in the short-term. The results obtained also showed that the uptake of other micronutrients such as Fe, Cu and Zn in the plants fertilized with the treatment containing Mn^{2+}:G7 (1:2) may be the outcome of a shuttle effect from this complex. Further studies including higher Mn doses of the Mn^{2+}:G7 (1:1 and 1:2) complexes applied to calcareous soil conditions, as well as biodegradability and microbial activity tests, would contribute to improve the knowledge of their reactivity in soil surfaces.

VI. Discusión general

En este capítulo se discuten los resultados más relevantes de esta tesis doctoral que tiene como propósito identificar los parámetros fisicoquímicos que influyen en la efectividad de agentes complejantes, tales como los lignosulfonatos (LS), heptagluconato (G7) y gluconato (G6) para su uso como fertilizantes de Fe, Mn y Zn. Si bien en cada uno de los capítulos de esta tesis se han discutido los resultados obtenidos, así como presentado conclusiones, en este apartado se realizará una discusión general que permite relacionar todos los aspectos descritos en cada uno de los capítulos anteriores y que suponen el avance científico de esta Tesis.

6.1 Capacidad complejante de los ligandos y su relación con los grupos funcionales y peso molecular

Los hallazgos obtenidos en el estudio de la MCC demostraron que tanto los LS, como el G7 y el G6 presentaron una mayor capacidad para complejar Fe^{3+} que para los metales divalentes (Fe^{2+}, Zn^{2+} y Mn^{2+}). Estos resultados han sido atribuidos a la mayor relación carga/radio iónico para el Fe^{3+} en comparación con el resto de metales divalentes (Smith y Martell, 1987). Los resultados de este trabajo demostraron también que la MCC es dependiente del tipo de ligando, los grupos funcionales presentes que contiene y su peso molecular. Los valores más altos de la MCC para Fe (III) los presentó el P4, un LS de bajo peso molecular y alto contenido de grupos fenólicos (Capítulo III, Tabla III.1 y III.2), así como por el G7 y G6 (Capítulo IV, Figura IV.1), cuyos ligandos también presentan un bajo peso molecular pero con un alto contenido de grupos hidroxilo. Estos resultados son consistentes con estudios previos (Pang *y col.*, 2008; Benedicto *y col.*, 2011; Rodríguez-Lucena *y col.*, 2011) donde LS modificados por ultrafiltración, fenolación o hidroximetilación presentaron una mejora en la capacidad de complejación. Por ello, es importante analizar la relación entre el contenido de grupos hidróxilo y la efectividad de los LS como fertilizantes, especialmente de los LS de pino que no han sido evaluados bajo condiciones agronómicas. Por lo tanto, sería interesante investigar la eficacia de nuevos LS modificados por hidroximetilación y de bajo peso molecular como fertilizantes de Fe.

El alto contenido de S-orgánico así como los grupos fenólicos y carboxílicos presentes en el E1 mejoró su capacidad de complejación de metales divalentes tales como Zn (II) y Fe (II) (Capítulo III, Tabla III.1 y III.2). Por otra parte, la mayor capacidad para complejar Mn (II) del P3 está relacionada solamente con el alto contenido de S-orgánico y grupos carboxílicos. Esta afinidad de los grupos sulfónicos con estos metales ha sido demostrada mediante espectros de FTIR (Capítulo III, Figura III.1c-e). Además, el alto contenido de S-orgánico presentado por los LS de eucalipto (E1) y

abeto (S1) en comparación con los LS de pino (Capítulo III, Tabla III.1), confirma la importancia de este grupo funcional en la capacidad para complejar Zn (II), resultando más baja para los LS de pino (Capítulo III, Tabla III.2). El P4, con bajo peso molecular y alto contenido de grupos fenólicos evaluado en este estudio, mostró una baja capacidad de complejación. Este hecho entra en contradicción con lo descrito por Benedicto *y col.* (2011), que relaciona una mayor capacidad complejante de Zn (II) por LS de eucalipto modificados por ultrafiltración y fenolación. Este resultado contradictorio podría relacionarse con el bajo contenido de S-orgánico para el P4.

Según los resultados obtenidos de la MCC, el G6 y el G7 mostraron una mayor capacidad para complejar Mn (II) y Fe (II), la cual está relacionada por la presencia de los grupos hidróxilo que permiten la formación de complejos estables a pH básico (Yamaguchi y Sawyer, 1985; Rodríguez-Lucena *y col*., 2010). Estos estudios han permitido confirmar también la mayor capacidad de complejación del G7 en comparación con el G6, independientemente del metal de estudio, estando de acuerdo con la hipótesis de una mayor estabilidad del G7 sugerida por Clemens y col. (1990), así como con la mayor estabilidad de un complejo comercial de Mn^{2+}:G7 en solución alcalina con impurezas de G6, frente a un complejo de Mn^{2+}:G6 (López-Rayo *y col.*; 2014).

6.2 Efecto del metal complejado y tipo de ligando en la estabilidad de los complejos

La legislación europea de fertilizantes (EU, 2003) indica que los productos basados en complejos de micronutrientes deben contener un mínimo del 5% de metal en la fracción soluble y un 80% de ésta debe estar en forma complejada. Estos parámetros también fueron determinados siguiendo el método oficial europeo para fertilizantes (CEN, 2011). La fracción complejada demostró ser dependiente del tipo de metal y ligando. Para los complejos de Fe (II) se obtuvo un porcentaje menor al 80% para la mayoría de los complejos con LS (Anexo I, Tabla S1) lo que indica una menor estabilidad que la de los complejos de Fe (III) que sí cumplen con esta norma. Además, estos resultados demuestran que estos complejos de Fe (III) fueron capaces de pasar a través de una membrana de filtro de 0.45 µm, sugiriendo una mayor solubilidad y disponibilidad del Fe para plantas crecidas en suelo calizo.

Este comportamiento diferencial es atribuido a la oxidación y consecuente formación de óxidos de Fe^{3+}, los cuales difieren a partir de la fuente de Fe usada (Fe (III) o Fe (II)) mostrados en la Figura VI.1. El espectro de Mössbauer de Fe^{3+}:G7 (1:2) (Capítulo

IV, Figura IV.3a) indica la presencia de oxi-hidróxidos de Fe (III) en coordinación octaédrica asociados a ferrihidrita. Por el contrario, el espectro de Fe^{2+}:G7 (1:2) (Capítulo IV, Figura. IV.3b) reveló la presencia de una fase ferrosa y una férrica probablemente asociado a la formación de óxidos verdes, los cuales pueden dar paso a la formación de lepidocrita, magnetita o goethita (Figura VI.1) bajo las condiciones de pH y temperatura empleadas en su preparación en el presente trabajo. De acuerdo con Loeppert y Clarke (1984), la ferrihidrita presenta una mayor velocidad de disolución en comparación con la lepidocrita, magnetita o goethita, lo cual puede explicar el mayor porcentaje de fracción complejada por los complejos de Fe (III) en comparación con los de Fe (II).

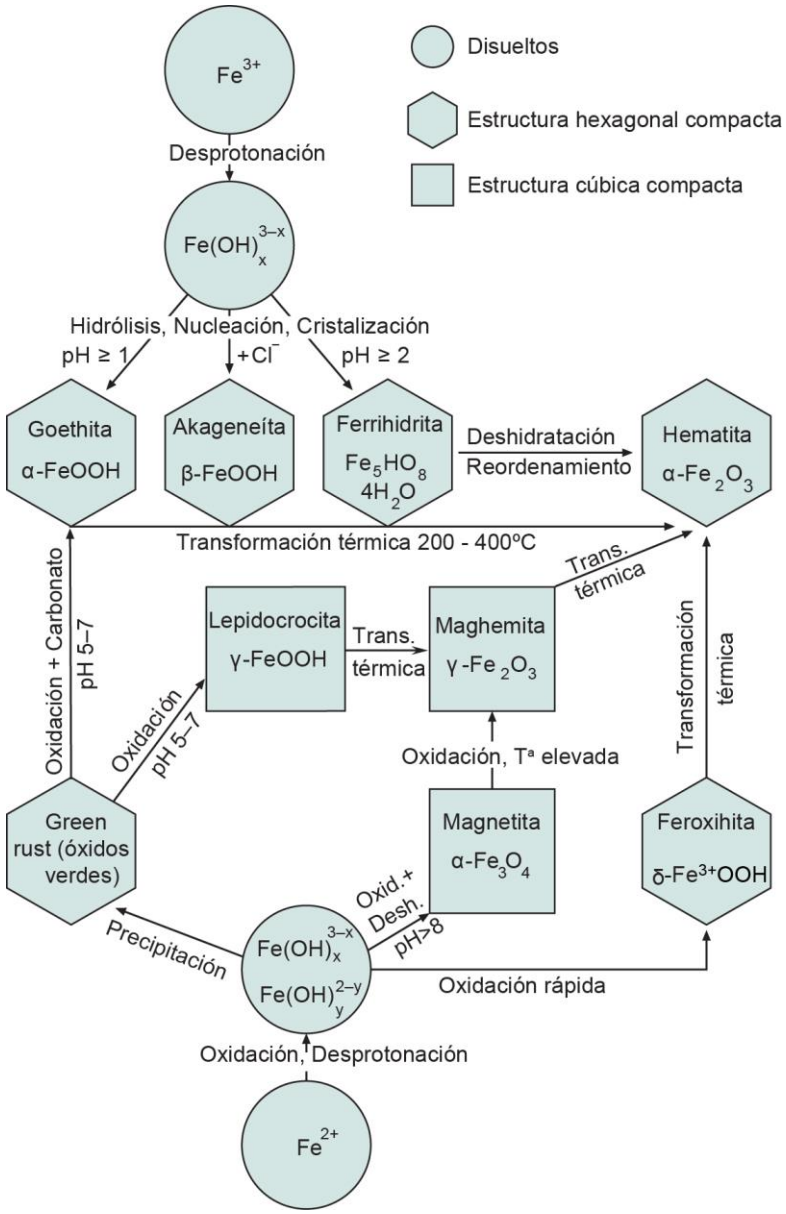

Figura. VI.1. Rutas de formación y transformación de los óxidos y oxi-hidróxidos de Fe. Fuente: Cornell y Schwertmann (2003).

Los complejos de Mn (II), tanto LS como el G7 y el G6 presentaron un elevado porcentaje de fracción complejada, satisfactoriamente superior al mínimo requerido. Esta mayor estabilidad presentada por todos los ligandos para mantener soluble el Mn (II) es consistente con estudios previos desarrollados en solución alcalina con complejos comerciales de estos agentes complejantes (López-Rayo *y col*. 2012, 2014). En este trabajo se demostró, además, que esta estabilidad es favorecida por la afinidad de Mn(II) con los grupos sulfónicos, hidróxilo y carboxílicos como se muestra en los espectros de FTIR de los Mn^{2+}:LS (Capítulo III, Figura III.1e) y en los complejos de Mn^{2+}:G6 y Mn^{2+}:G7 (Capítulo V, Figura V.1a-b). Igualmente la fracción complejada de Zn (II) por los LS fue mayor al mínimo establecido, demostrando también así la estabilidad de los complejos de Zn (II) a partir de LS de pino a pH alcalino. Esta estabilidad ha sido descrita previamente para complejos de Zn (II) con LS en solución alcalina (López-Rayo *y col*., 2012; Lucena *y col*., 2010). Benedicto *y col*. (2011) también confirmaron que un LS modificado por sulfonación es capaz de formar complejos más estables con Zn (II) a pH 9 en comparación con los LS de eucalipto modificados por fenolación o ultrafiltración. Por otra parte, estos autores también demostraron que los LS de eucalipto modificados por fenolación o ultrafiltración permiten una mayor complejación de Zn y la formación de complejos relativamente débiles, los cuales promovieron una mejor absorción y translocación de Zn^{2+} a las plantas por aplicación foliar a pH 5.

El lignosulfonato de magnesio (P4) con bajo peso molecular y alto contenido de grupos fenólicos presentó una baja estabilidad para complejar Zn (II) a pH 9 (Capítulo III, Tabla III.1 y III.2), observándose su precipitación durante la preparación del complejo a pH 6, lo cual impidió su formación y en consecuencia la evaluación adecuada de su fracción complejada. La precipitación del LS durante la preparación del complejo podría ser atribuida al bajo contenido de grupos sulfónicos contenidos en comparación con los LS de eucalipto modificados, ya que estos grupos funcionales muestran una mayor interacción con Zn (II) a pH 6 (Capítulo III, Figura III.1d). La afinidad del Zn^{2+} por los grupos sulfónicos en comparación con los grupos carboxílicos ionizados a pH 6 se confirma con las constantes de estabilidad presentadas por ambos grupos (Tabla VI.1). Además de los parámetros sugeridos por Benedicto *y col*. (2011), de acuerdo a los resultados obtenidos en esta Tesis se sugiere que un alto contenido de grupos sulfónicos es un parámetro esencial que influye en la eficacia de los LS como fertilizantes de Zn (II), el cual les permitiría mantener el Zn soluble en soluciones con un pH cercano a la neutralidad (como las usadas en aplicación foliar o hidroponía).

Tabla VI.1. Constantes de estabilidad Log Kº (X + Y ⇆ XY) de los complejos 1:1.

Catión (X)	Ligando (Y)		
	SO_4^{2-}	$R\text{-}COO^{-}$ *	OH^-
H^+	1.99	4.76	13.98
Fe^{3+}	4.25	4.24	12.0
Fe^{2+}	2.39	1.40	4.60
Mn^{2+}	2.25	1.40	3.40
Zn^{2+}	2.34	1.57	5.00
Cu^{2+}	2.36	2.21	6.50

Datos obtenidos de NIST (Smith y Martell, 2004). *Los datos para la complejación del grupo carboxilo han sido estimados a partir del acetato.

6.3 Fraccionamiento de complejos para evaluar la biodisponibilidad de los micronutrientes en condiciones de hidroponía y suelo calizo.

Los resultados discutidos previamente demuestran que es necesario estudiar, además de la fracción complejada, algunos parámetros adicionales como el peso molecular. Por lo tanto, el fraccionamiento de complejos por GFC y HPSEC fue el punto de partida para evaluar la efectividad de los complejos como fertilizantes. Ambos métodos de fraccionamiento demostraron la formación de compuestos de HMW para todos los complejos de Fe^{3+}:LS (Capítulo III, Figura III.2 y III.3), así como para los complejos de Fe^{3+}:G7 por GFC (Capítulo IV, Figura IV.3d-f). Esto fue confirmado por la formación de complejos polinucleares de Fe^{3+} mostrados en el análisis por Mössbauer (Capítulo IV, Figura IV.3a). En estos complejos polinucleares existen formaciones tetraédricas y/o octaédricas de Fe^{3+} rodeadas de grupos hidróxilos, las cuales serían estabilizadas por los grupos carboxilos y fenólicos en el caso de los LS, mientras para el G6 y G7 por los grupos hidróxilo y carboxilo, tal como se ha descrito para los ácidos húmicos (Kulikova *y col.*, 2017; Cieschi *y col.*, 2019).

El fraccionamiento de los LS por HPSEC a pH 9 demostró que éstos presentan una mayor capacidad de complejación de Fe(III) a este pH que a pH 6 (Capítulo III, Figura III.3). Pang *y col.* (2008) propusieron que este fenómeno es debido a la expansión de los LS a pH 9 mejorando su capacidad complejante en comparación con el pH 6 donde el LS se contrae. A su vez, la expansión es consecuencia de la ionización de los grupos carboxilos, fenólicos y sulfónicos a pH 9, mientras que a pH 6 solamente

son ionizados los grupos carboxilos y sulfónicos (Yan *y col*., 2010). Además, esta mayor desprotonación incrementa la proporción de grupos donadores de electrones capaces de enlazarse a los metales.

Por otra parte, este fenómeno de expansión y contracción química, dependiente de la presencia de grupos funcionales, puede explicar el menor porcentaje de Fe recuperado por el P4 después de su fraccionamiento por GFC a pH 6, en comparación con el P2 que presentó un alto porcentaje de recuperación debido a su alto peso molecular y mayor contenido de grupos carboxílicos. Por consiguiente, es posible hipotetizar que los complejos del P4 resultarían fertilizantes efectivos en hidroponía debido a su baja estabilidad, mientras que los de P2 podrían ser aplicados a nivel foliar o en condiciones de suelo calizo, donde el uso de complejos más estables es imperativo (Carrasco *y col*., 2012).

Para los complejos de Fe^{2+}:LS, ambos métodos de fraccionamiento demostraron la distribución del Fe, tanto en la fracción de HMW como en la fracción de LMW. Esta distribución de Fe en ambas fracciones es consistente con la afinidad de Fe^{2+} tanto por sitios fuertes (carboxílicos y fenólicos) como por débiles (grupos sulfato) descrita por (Smith *y col*., 2002). Sin embargo, el fraccionamiento de los Fe^{2+}:LS por GFC permitió confirmar que el Fe está asociado mayormente con la fracción de LMW (<100 kDa) para la mayoría de los complejos (Capítulo III, Tabla III.3). Por lo tanto, el Fe unido a los sitios de complejación débiles fue encontrado en la fracción de LMW, mientras que en la fracción de HMW fue encontrado el Fe enlazado a los sitios de complejación fuertes, de acuerdo con Wu *y col*. (2004). Esta interacción de los complejos formados con Fe (II) y grupos débiles (grupos sulfato) fue confirmada por los espectros de FTIR a pH 6 (Capítulo III, Figura III.1c) y se corresponde con la mayor estabilidad mostrada en comparación con los grupos carboxílicos (Tabla VI.1).

Adicionalmente, el fraccionamiento por GFC de los complejos de Fe^{2+}:LS también demostró una menor estabilidad en comparación con los complejos de Fe^{3+}:LS, ya que menos del 73% de Fe fue recuperado para la mayoría de los complejos de Fe (II) (Capítulo III, Tabla III.3). Por lo tanto, estos resultados son consistentes con los obtenidos en la fracción complejada, donde se preparan complejos de LS, G7 y G6 más estables a partir de una fuente de Fe (III).

El fraccionamiento por GFC y por HPSEC a pH 6 reveló la asociación de Zn^{2+} y Mn^{2+} con la fracción de LMW para los complejos con LS (Capítulo III, Tabla III.3 y III.4). La formación de complejos de bajo peso molecular (<700 Da) de G6 y G7 con Mn^{2+} (Capítulo V, Figura V.1c-d) también se observó por GFC. Este hecho es atribuido a

que los metales con una fuerza de unión débil se encuentran principalmente en las fracciones de LMW de materia orgánica disuelta (Wu *y col.*, 2004), como ha sido demostrado para Zn^{2+} y Mn^{2+} distribuidos en la fracción de LMW de materia orgánica coloidal (Worms *y col.*, 2010). Este hecho podría explicar el menor porcentaje de metal recuperado para estos complejos por GFC (Capítulo III, Tabla III.3), así como el menor porcentaje de área de pico presentado por estos complejos en HPSEC (Capítulo III, Tabla III.4). El fraccionamiento de LS por HPSEC también señaló que E1 presentó el porcentaje de área de pico más bajo para ambos metales, mientras S1 presentó el más alto, a ambos pHs. Estos resultados mostraron una tendencia similar a la capacidad de E1 para proveer Zn^{2+} a plantas de judía por aplicación foliar o en suelo calizo, debido a su relativa baja estabilidad y bajo peso molecular, respectivamente (Benedicto *y col.*, 2011; Cieschi *y col.*, 2016). De igual manera, S1 presentó alto contenido de LSA, alto peso molecular y bajo contenido de grupos carboxílicos (Capítulo III, Tabla III.1). Estas características son consistentes con la mayor capacidad de complejación para el producto S1 sin fraccionar (Martín-Ortiz *y col.*, 2009) y su baja efectividad para proveer Zn^{2+} bajo condiciones de suelo calizo y aplicación foliar.

El fraccionamiento por HPSEC de los complejos de Mn (II) a pH 9 mostró un ligero incremento en la fracción de HMW comparado con el observado en la fracción de LMW a pH 6 discutido previamente. Este resultado sugiere que la asociación de Mn^{2+} con las fracciones de LS es dependiente del pH en el que los complejos son preparados. A pH 9 es posible formar complejos más estables como consecuencia de la mayor ionización de los grupos fenólicos, carboxílicos y sulfónicos como describió Yan *y col.* (2010). Esto explica el mayor porcentaje de fracción complejada que presentan los LS, G6 y G7 con Mn a pH 9, en comparación con la baja estabilidad observada para los complejos de G6 y G7 a pH 6 (Capítulo V, sección 5.3.2).

La afinidad del Mn^{2+} por los grupos sulfónicos y carboxílicos observada en este estudio, es coherente con los resultados obtenidos por Martín-Ortiz *y col.* (2016) acerca de la capacidad de un fertilizante de NPK recubierto por un LS de eucalipto con un alto contenido de S y grupos carboxílicos, el cual fue capaz de proveer Mn a plantas de trigo bajo condiciones de hidroponía y de suelo calizo. Por lo tanto, entre los LS estudiados, es posible sugerir que P3 puede ser un fertilizante efectivo para aportar Mn de acuerdo a su mayor capacidad de complejación (Capítulo III, Tabla III.2). Esto sería debido por su mayor contenido de S-orgánico, en comparación con la mayoría de LS de pino, y su mayor contenido en grupos carboxílicos comparado con S1 y E1.

6.4 Influencia de la relación metal:ligando en la formación de complejos y su capacidad para proveer Fe o Mn

Los resultados previamente discutidos sobre la caracterización y fraccionamiento de los complejos por GFC y HPSEC han resultado de suma importancia para la identificación de los parámetros que influyen en la estabilidad de los complejos. Entre ellos, es posible destacar: **1)** el tipo de ligando que interviene en la capacidad de complejación y estabilidad del complejo; **2)** los grupos funcionales presentes en el ligando y su peso molecular, que influyen en la afinidad por el metal; **3)** el metal complejado que favorece la formación de compuestos de HMW o LMW, así como la formación de óxidos con mayor o menor velocidad de disolución en el caso del Fe; y **4)** la variación del pH que interviene en la ionización de los grupos funcionales del ligando.

Por otra parte, varios autores (Stevenson, 1982; García-Mina, 2006; Rodríguez-Lucena *y col.*, 2010; Carrasco *y col.*, 2012) han demostrado que la relación metal:ligando influye en la presencia de diferentes sitios de coordinación y, en consecuencia, en la formación de complejos de estabilidad variable, siendo las relaciones metal:ligando bajas las que favorecen la formación de complejos más estables. Por lo tanto, el siguiente paso a seguir fue la evaluación de la influencia de la relación metal:ligando para los ligandos biodegradables G7 y G6, en su eficacia como fertilizantes de Fe o Mn bajo condiciones de hidroponía y suelo calizo. La caracterización química y estructural de los complejos de G6 y G7, también ha permitido sugerir las posibles estructuras que se formarían para los complejos con relaciones 1:1 y 1:2 (Capítulo V, Figura V.3), las cuales influirían en la estabilidad de los diferentes ligandos.

6.4.1 Efecto del tipo de ligando en la estabilidad del complejo y disponibilidad del micronutriente

La evaluación de la efectividad de los tratamientos de Fe y Mn bajo condiciones de hidroponía y suelo calizo proporcionó resultados que fueron consistentes con los obtenidos en la caracterización, confirmando así que el tipo de ligando es uno de los parámetros importantes para la efectividad de los complejos. En todos los experimentos se observó que el G6 presentó una eficacia menor para proveer micronutrientes en comparación con G7. Esta diferencia fue más destacable para Fe en condiciones de suelo calizo, tanto para el tratamiento Fe^{3+}:G6 (1:1) (Capítulo IV, Tabla IV.3) como para el Mn^{2+}:G6 (1:1) (Capítulo V, Tabla V.4), presentando ambos

tratamientos concentraciones de Fe en hoja más bajas que las de los tratamientos equimolares con G7. La aplicación del tratamiento Fe^{3+}:G6 (1:1) también demostró una disminución en las concentraciones de Mn, Cu y Zn a largo plazo (Capítulo IV, Tabla IV.4) en comparación con el tratamiento Fe^{3+}:G7 (1:1) que presentó concentraciones más altas en la mayoría de los tejidos de las plantas estudiadas. La baja eficiencia de los complejos con G6 se relaciona con la baja capacidad complejante de G6 con Fe (Capítulo IV, Figura IV.1) y Mn (Capítulo V, sección 5.3.1), así como por la baja estabilidad de sus complejos previamente señalada por varios autores a pH alcalino (Clemens *y col.*, 1990; López-Rayo *y col.*, 2012, 2014, 2015).

Considerando que G6 y G7 presentan los mismos grupos funcionales, la formación de sus complejos con Fe o Mn en relaciones equimolares fue asociada a la interacción del metal con hidróxilos secundarios y el grupo carboxilo observada en los complejos equimolares de Mn por FTIR (Capítulo V, Figura V.1). Estos resultados son coherentes con estudios previos (Yamaguchi y Sawyer, 1985; van Duin *y col.*, 1989) que han demostrado la interacción de metales tanto con el grupo carboxilo como con el hidróxilo secundario localizado en la posición α de los ligandos, formando un anillo de cinco miembros. Sin embargo, la baja estabilidad de los complejos de G6 observada en nuestro estudio, podría ser atribuida a las diferencias en las estructuras de los ligandos. Por lo tanto, las estructuras de los complejos obtenidas a partir de la isomería de cada ligando, sugirió que el complejo de G7 a parte de formar un anillo de cinco de miembros con el Mn, este también puede ser estabilizado por el hidróxilo primario, mientras que en el complejo de G6 su conformación no permitiría esta unión, explicando su menor estabilidad.

6.4.2 Mecanismos químicos implicados en la absorción de Fe y Mn por la planta, en base a la relación metal:ligando

Aunque el tipo de ligando resultó ser un parámetro importante en la eficacia de los fertilizantes como se ha descrito anteriormente, el resultado con mayor impacto de este trabajo fue el estudio y aplicación de los complejos con diferentes relaciones metal:ligando para Fe y Mn. En este sentido, los resultados fueron más notorios en los tratamientos de Fe^{3+}:G7 que en Mn^{2+}:G7 debido a la mayor cantidad de ligando adicionada, como consecuencia de la mayor capacidad complejante de G7 con Fe que la obtenida para Mn. En la Figura VI.2 se intentan recoger los mecanismos químicos observados para las diferentes relaciones metal:ligando aplicadas en condiciones de hidroponía y suelo calizo. Así: **1)** Cuando se aplicó el complejo de Fe^{3+}:G7 relación 1:3, se observó una disminución en la absorción de micronutrientes e incluso en la

absorción de Fe (Capítulo IV, Tabla IV.3 y IV.4), hecho que fue relacionado con el desplazamiento del equilibrio en la reacción de complejación hacia la formación de complejos, como consecuencia del exceso de ligando; **2)** La aplicación del complejo de Fe^{3+}:G7 relación 1:0.5 implicó un descenso en la translocación del Fe a causa del bajo contenido de ligando causado por el desplazamiento del equilibrio de la reacción de complejación hacia la liberación de Fe^{3+}, ocasionando su precipitación como oxihidróxidos de Fe en la superficie de la raíz (Capítulo IV, Tabla IV.1) debido al alto pH en el medio; **3)** Por el contrario, las relaciones 1:1 y 1:2 de Fe^{3+}:G7 promovieron una mejor absorción de Fe y el equilibrio entre los micronutrientes como consecuencia de la estabilidad entre el Fe^{3+} y el ligando que se obtiene en estas relaciones molares, evitando la precipitación de oxihidróxidos de Fe.

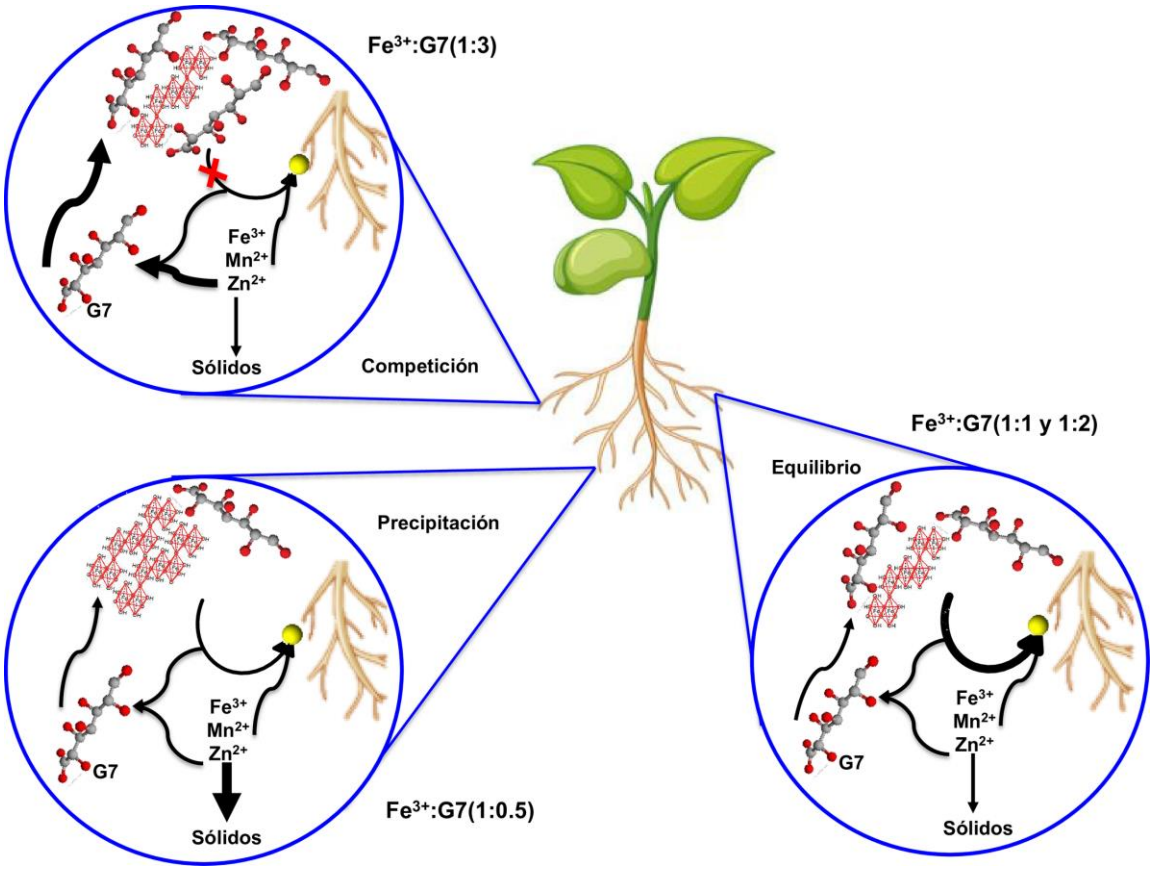

Figura VI.2. Mecanismos químicos de la relación metal:ligando. Fuente: Propia.

Este mecanismo propuesto para la relación 1:1 de Fe^{3+}:G7 también es coherente con la mejora en la absorción de micronutrientes distintos al aportado por este complejo (Capítulo IV, Tabla IV.2 y IV.4), observado en el incremento de estos en la fracción disponible del suelo (Capítulo IV, Tabla IV.3). Además, el tratamiento Fe^{3+}:G7 (1:2) presentó un efecto tan rápido como el del EDTA para aportar Fe a las plantas bajo condiciones de suelo calizo (Capítulo IV, Tabla IV.3). Cuando esta relación fue

aportada como un complejo de Mn^{2+}, no sólo incrementó el contenido de Mn en hoja a los 7 DAT, sino también el contenido de Fe (Capítulo V, Tabla V.4) y la fracción soluble de Fe (Capítulo V, sección 5.3.4). Así, ambos experimentos indicaron un efecto rápido del complejo de relación 1:2 para proveer Fe, sugiriendo un efecto de recarga de esta relación cuando es aplicada en suelo calizo. Este resultado también puede ser consecuencia de la formación de oxi-hidróxidos de Fe^{3+} observados en el espectro de Mössbauer de la relación 1:2 (Capítulo IV, Figura IV.3a), consistentes con ferrihidrita. De acuerdo con Loeppert y Clarke (1984), este óxido de Fe amorfo presenta una mayor velocidad de disolución de Fe en comparación con otros oxi-hidróxidos de Fe (Figura VI.1), explicando su efecto rápido para proveer Fe a las plantas observado en nuestro estudio bajo condiciones de suelo calizo. Por el contrario, los complejos con las relaciones 1:0.5 y 1:3 (Fe^{3+}:G7) no fueron suficientes para proveer Fe a plantas de tomate en condiciones de hidroponía (Capítulo IV, Tabla IV.1) y plantas de soja en condiciones de suelo calizo (Capítulo IV, Tabla IV.3). Estos resultados concuerdan con varios autores (Stevenson, 1982; Garcia-Mina, 2006), que han demostrado que la estabilidad de los complejos a pH alcalino es mayor a bajas relaciones de Fe:sustancias húmicas que a altas relaciones (p. ej., 1:1 y 1:2 en comparación con 1:0.5 en este estudio), ya que el incremento de la relación metal:ligando reduce los grupos funcionales libres.

Por lo tanto, en el caso del complejo de Fe^{3+}:G7 relación 1:3 (que también es una baja relación) se esperaría una mayor estabilidad de este complejo. Sin embargo, nuestros estudios indican todo lo contrario, como consecuencia del mecanismo propuesto para esta relación (Figura VI.2) consistente con la disminución de micronutrientes absorbidos por las plantas cuando se aplica un exceso de ligando, como se ha descrito previamente para DTPA, BPDS y EDTA (Lindsay y Schwab, 1982; Wallace *y col.*, 1983). Un efecto similar fue observado para la relación 1:3 y 1:0.5 de Mn^{2+}:G7, las cuales presentaron una menor capacidad para proveer Mn a las plantas en comparación con la relación 1:1 y 1:2 bajo condiciones de hidroponía y suelo calizo.

De esta manera, los resultados previamente discutidos confirman la importancia de la relación metal:ligando en la eficacia de los complejos como fertilizantes, siendo la relación 1:1 y 1:2 de complejos a base de G7, las alternativas más prometedoras en sustitución de quelatos sintéticos como el HBED y el EDTA.

Estas diferencias encontradas en la absorción de micronutrientes aportados en forma de complejos de Fe^{3+}:G7 y Mn^{2+}:G7 también dependieron de las condiciones experimentales en las que fueron aplicados. En condiciones de hidroponía, todos los

complejos resultaron igual de eficientes para proveer Fe (Capítulo IV, Tabla IV.1) y Mn (Capítulo V, Figura V.2b). Como consecuencia, todos los tratamientos presentaron una relación Fe:Mn en hoja dentro del intervalo 1.5-2.5 establecido por Adriano (2001), así como un incremento en el índice clorofila en comparación con las plantas deficientes de Fe (Capítulo IV, Figura IV.4) y Mn (capítulo V, Figura V.2a). No obstante, el incremento en el peso seco (DW) de la raíz fue un indicador de la mejor capacidad de la relación 1:1 y 1:2 para proveer Fe (Capítulo IV, Tabla IV.1) y Mn (Capítulo V, Tabla V.1) bajo condiciones de hidroponía (Marschner, 2012; Ejraei, 2013; Carrasco-Gil *y col.*, 2016; López-Rayo *y col.*, 2016).

En condiciones de suelo calizo, la relación entre el índice de clorofila y la concentración de micronutriente en hoja solo se observó para los complejos de Fe^{3+}:G7. En este sentido, la relación fue eficaz a corto plazo para el Fe^{3+}:EDTA con el Fe proporcionado por el quelato (Capítulo IV, Figura IV.5a), mientras que en el caso de los complejos solo a largo plazo (Capítulo IV, Figura IV.5b), probablemente como consecuencia de la formación de compuestos de HMW para los complejos de Fe (III) (Capítulo IV, Figura IV.3a). La ausencia de relación entre el índice de clorofila y el contenido de Mn en suelo, es coherente con lo señalado por Marschner (2012), que indica que la reducción en la concentración de clorofila se observa únicamente en condiciones de deficiencia severa de Mn. No obstante, durante todo el estudio, todos los tratamientos presentaron concentraciones de Mn en hoja superiores al intervalo crítico de deficiencia (Jones, 2012). El incremento de Mn en las plantas a corto plazo (Capítulo V, Tabla V.3) fue consecuencia del efecto de los tratamientos de Mn aplicados, siendo las relaciones 1:1 y 1:2 de Mn^{2+}:G7 las que presentaron una eficacia similar al Mn^{2+}:EDTA para proveer Mn a las plantas. A largo plazo este incremento fue motivado por el antagonismo causado por la deficiencia de Fe (Capítulo V, Tabla V.4), así como a una mejor respuesta en la acidificación de la rizósfera por parte de la soja (Marschner, 1988; Martín-Ortiz *y col.*, 2016), provocando un comportamiento similar en todos los tratamientos. En el caso de la relación Fe/Mn en hoja, los valores fueron más bajos que en hidroponía, incluso para plantas que demostraron un balance adecuado de Fe y Mn. Estos resultados sugieren que el rango establecido (1.5-2.5) para evaluar la nutrición adecuada de complejos, puede ser algo elevado para experimentos en condiciones de suelo calizo. Sin embargo está claro que valores menores a 0.4 indican un desequilibrio entre ambos micronutrientes (Ghasemi-Fasaei *y col.*, 2002). Estos valores se obtuvieron para los tratamientos de Mn a largo plazo (Capítulo V, Tabla V.4), los cuales presentaron una relación Fe:Mn $\leq$ 0.32, como consecuencia de la deficiencia de Fe obtenida en las plantas.

La baja concentración de Fe en hoja y de otros micronutrientes como Cu y Zn (Capítulo V, Tabla V.4) obtenida tras la aplicación de los complejos de Mn, a largo plazo, es atribuida a la baja dosis de metal:ligando aplicada. Aunque la concentración obtenida de estos micronutrientes se encontraba por debajo del rango crítico de deficiencia (Jones, 2012), la relación 1:2 de Mn^{2+}:G7 promovió un incremento de Zn y Cu en tallo, en comparación con las plantas deficientes de Mn. Este incremento pudo ser debido a un efecto de recarga de este complejo en el medio similar al presentado por el Mn^{2+}:EDTA (1:1). Probablemente este efecto no se trasladó a hoja como consecuencia de la baja dosis aplicada. Estos resultados son coherentes con los obtenidos en los experimentos donde se aplicó Fe. En estos, la concentración de complejos adicionada fue mayor, observándose un incremento en la absorción de Fe, Mn, Cu y, en menor proporción, Zn (Capítulo IV, Tabla IV.3 y IV.4) tras la aplicación de Fe^{3+}:G7 (1:1) y (1:2) a largo plazo.

Por lo tanto, estos resultados indicaron que la efectividad de ligandos biodegradables en suelo calizo no solo depende del ligando y la relación metal:ligando, sino también de la dosis de complejo aplicada. Por tanto, se debería contemplar un incremento en la dosis y frecuencia de aplicación cuando se utilizan agentes complejantes como fertilizantes aplicados al suelo.

VII. CONCLUSIONES

Las conclusiones que se extraen de los resultados de esta Tesis doctoral, tras la caracterización, fraccionamiento y realización de ensayos biológicos de complejos metálicos con lignosulfonatos, heptagluconato y gluconato son:

1.- Los lignosulfonatos, heptagluconato y gluconato han mostrado una mayor capacidad para complejar Fe^{3+} que para cationes divalentes (Fe^{2+}, Mn^{2+} y Zn^{2+}).

El bajo peso molecular, los grupos hidróxilos presentes en gluconato y heptagluconato, así como los grupos fenólicos presentes en los lignosulfonatos son los factores más importantes en la complejación de Fe^{3+}. Por lo tanto, se recomienda profundizar en el estudio de lignosulfonatos modificados por hidroximetilación.

2.- Las técnicas de fraccionamiento por GFC y HPSEC fueron herramientas útiles para analizar la distribución del peso molecular de complejos de Fe, Mn y Zn, siendo un análisis relevante para predecir la efectividad de los complejos como fertilizantes.

La formación de complejos polinucleares de alto peso molecular a partir de una fuente de Fe^{3+} puede explicar su disponibilidad a largo plazo cuando los complejos se aplican como fertilizantes.

Los complejos de Mn (II) y Zn (II) forman especies discretas, promoviendo un mejor desplazamiento y absorción por las plantas.

El P4, un lignosulfonato con un alto contenido de grupos fenólicos y bajo peso molecular, así como el P2 con bajo contenido de S-orgánico serían fertilizantes adecuados para proveer Fe, mientras el P3 con alto contenido de S-orgánico y grupos carboxílicos sería un fertilizante eficiente de Mn.

3.- Se han podido establecer factores que inciden en la eficacia de los complejos de Fe y Mn para nutrir los cultivos.

En concreto se han mostrado relevantes:

El tipo de ligando. Se ha demostrado una mayor efectividad del heptagluconato para proveer Fe y Mn a las plantas en comparación con el gluconato bajo condiciones de hidroponía y suelo calizo. El heptagluconato puede ser una alternativa biodegradable al empleo de quelatos de HBED y EDTA.

El estado de oxidación del Fe. Los complejos a partir de Fe(III) presentan formas polinucleares de Fe(III), lo cual influye en la mejor disponibilidad de Fe a las plantas, respecto a los complejos formados a partir de Fe (II).

La relación metal:ligando en el heptagluconato. Las relaciones 1:1 y 1:2 son más eficaces para proveer Mn y Fe, mientras que las relaciones 1:3 y 1:0.5 limitan la absorción o solubilidad de los micronutrientes.

Dosis. Es necesario incrementar la dosis y frecuencia de complejos con agentes biodegradables de heptagluconato y gluconato cuando se aplican como fertilizantes en suelo.

CONCLUSIONS

After the characterization, fractionation and evaluation of biological assays of the metallic complexes with lignosulfonates, heptagluconate and gluconate, the conclusions generated from this doctoral thesis were:

1.- The lignosulfonates, gluconate and heptagluconate showed a higher complexing capacity for Fe^{3+} compared to divalent metals (Fe^{2+}, Mn^{2+} and Zn^{2+}).

The most important factors for the Fe^{3+} complexation by the ligands was their low molecular weight, as well as the phenolic groups in the lignosulfonates and the hydroxyl groups present in the G7 and the G6. Therefore, it would be necessary to further studies focus on the evaluation of lignosulfonates modified by hydroxymethylation.

2.- The fractionation techniques of GFC and HPSEC were useful tools to analyze the molecular weight distribution of Fe, Mn and Zn complexes, being a relevant analysis to predict the effectiveness of the complexes as fertilizers.

The formation of high molecular weight polynuclear complexes from Fe^{3+} source may explain its long-term availability when those were applied as fertilizers.

The Mn(II) and Zn(II) complexes form discrete species, promoting their displacement and uptake by the plants.

The P4, a lignosulfonate with a high content of phenolic groups and low molecular weight, as well as the P2 with low content of sulphur organic would be adequate Fe fertilizers, whereas the P3 with a high content of sulphur organic and carboxylic groups would be an efficient Mn fertilizer.

3.- The factors that influence the efficacy of the Fe and Mn complexes to nourish crops have been established.

In particular, it has been shown relevant:

The ligand type. The heptagluconate presented a higher efficacy to provide Fe and Mn to the plants as compared to the gluconate under hydroponics and calcareous soil conditions. Thus, the G7 may be a biodegradable alternative to the employment of synthetic chelates such as HBED y EDTA.

The iron oxidation state. The Fe(III) complexes present Fe(III) polynuclear structures, which influence in the better Fe availability to the plants compared to the complexes formed from the Fe (II) source.

The metal:ligand ratio of the heptagluconate. The 1:1 and 1:2 ratios were able to provide Mn and Fe to the plants while the 1:3 and 1:0.5 limited the uptake or solubility of the micronutrients.

Dose. An increase in the dose and frequency of the complexes is recommended in soil when biodegradable complexing agents as gluconate and heptagluconate are applied.

VIII. BIBLIOGRAFÍA

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IX. Anexo I

ASSESSING METAL–LIGNOSULFONATES AS FERTILIZERS USING GEL FILTRATION CHROMATOGRAPHY AND HIGH-PERFORMANCE SIZE EXCLUSION CHROMATOGRAPHY

Table III.S1. Characteristics of products used in the laboratory. Values are given as means ± standard error (*n*=3).

Sample	Complexed element (g/Kg^{-1} DW)	Soluble element (g/Kg^{-1} DW)	% complexed fraction
Fe(III):P1	86.2 ± 0.6	70.0 ± 1.1	124
Fe(III):P2	79.0 ± 2.2	91.0 ± 0.1	89.0
Fe(III):P3	99.1 ± 9.6	87.2 ± 2.9	114
Fe(III):P4	102 ± 0.2	108 ± 3.2	95.1
Fe(III):P5	90.4 ± 1.0	70.4 ± 1.0	128
Fe(II):P1	27.7 ± 2.0	40.3 ± 0.2	68.6
Fe(II):P2	46.7 ± 1.7	44.5 ± 0.9	105
Fe(II):P3	48.3 ± 1.1	55.4 ± 6.0	87.2
Fe(II):P4	24.6 ± 1.4	32.4 ± 3.9	75.8
Fe(II):P5	35.4 ± 0.8	44.6 ± 0.4	79.4
Zn(II):P1	63.8 ± 0.4	64.9 ± 1.0	98.2
Zn(II):P2	68.4 ± 1.9	71.5 ± 3.1	95.6
Zn(II):P3	73.8 ± 0.6	79.1 ± 2.6	93.3
Zn(II):P5	72.8 ± 1.7	82.9 ± 1.3	87.8
Mn(II):P1	81.1 ± 1.6	88.0 ± 2.6	92.2
Mn(II):P2	89.4 ± 5.5	93.2 ± 2.6	96.0
Mn(II):P3	104 ± 1.3	108 ± 3.6	96.8
Mn(II):P5	84.4 ± 5.1	95.5 ± 2.3	88.4

Table III.S2. The metal soluble measured by 0.22 μm filtration of the complexes from pine LS (P2), spruce LS (S1) and eucalyptus LS (E1) adjusted to pH 6 and pH 9. Values are given as means ± standard error (*n*=3).

Sample	metal content (mg L^{-1})	
	pH6	pH9
Fe(III):P2	35.9 ± 8.4	125 ± 5.5
Fe(II):P2	106 ± 3.2	109 ± 15
Zn(II):P2	79 ± 1.2	79.7 ± 2.4
Mn(II):P2	130 ± 1.3	139 ± 0.9
Fe(III):S1	0.2 ± 0.1	0.8 ± 0.1
Fe(II):S1	4.9 ± 0.3	2.2 ± 0.4
Zn(II):S1	5.1 ± 0.1	4.9 ± 0.1
Mn(II):S1	5.9 ± 0.0	5.4 ± 0.3
Fe(III):E1	0.8 ± 0.1	1.8 ± 1.4
Fe(II):E1	1.1 ± 0.4	0.5± 0.1
Zn(II):E1	6.4 ± 0.2	5.9 ± 0.4
Mn(II):E1	7.1 ± 0.3	6.5 ± 0.6

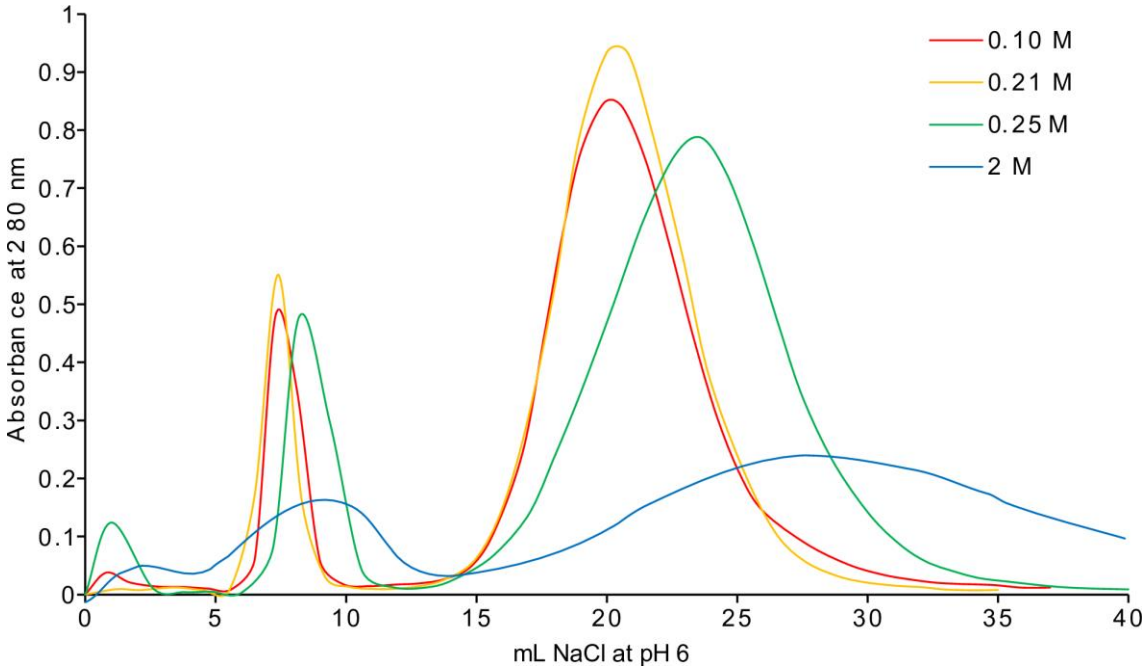

Figure III.S1 Mobile phases tested for the fractionation of pine lignosulfonate from gel filtration chromatography.

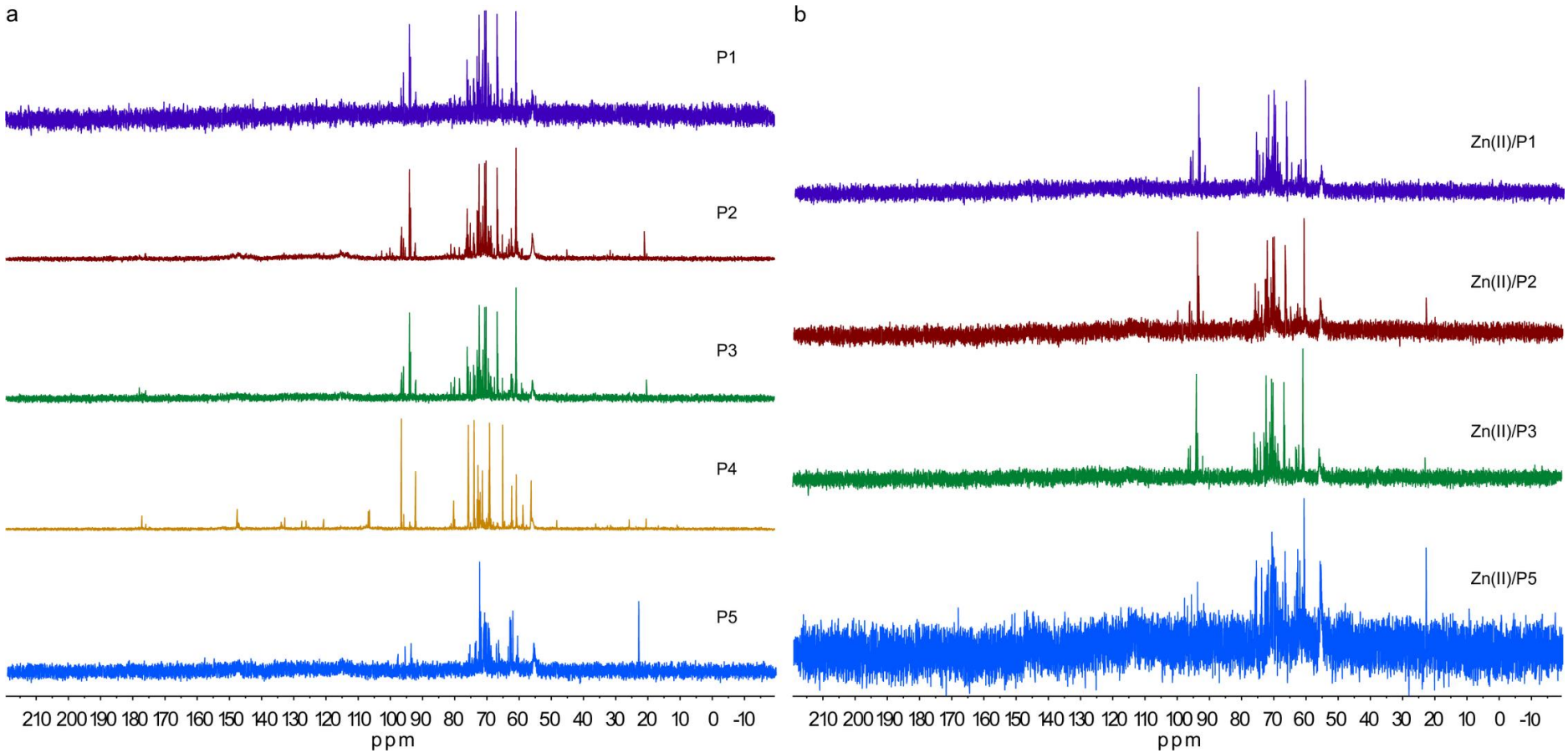

Figure III.S2 ^{13}C-nuclear magnetic resonance spectra of pine LS obtained from different sulfite pulping processes a) and their Zn (II)/LS complexes b).

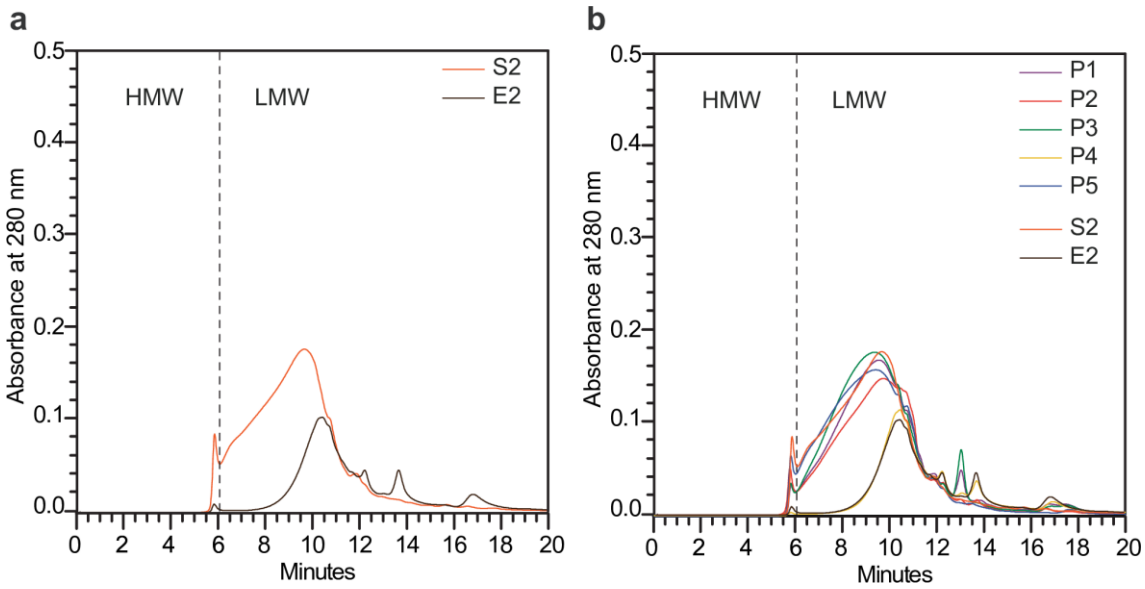

Figure III.S3 Elution profiles obtained by HPSEC on Biosep Sec-S2000 column using 0.1 M lithium chloride in 90 mM tetrabutylammonium hydroxide. Chromatograms were obtained for a) the standard lignosulfonates and b) pine lignosulfonate along with the standard lignosulfonates for comparison of molecular weight distribution. The average molecular weight of S2 and the E2 are around 52000 Da and 18000 Da, respectively. The blue dextran exclusion time is shown in dotted line.