Analytical technologies to study the biological and environmental implications of iron-fertilisation using synthetic ferric chelates: the case of Fe(III)-EDDHA – a review

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

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

Iron-fertilisation is the best and most commonly used technique to correct for Fe-deficiency in established fruit tree orchards. The active ingredients can be either inorganic or organic Fe-containing compounds. Foliar fertilisation with inorganic Fe compounds (e.g., FeSO₄) or some organic Fe complexs, including natural (e.g., citrate) and synthetic ligands such as ethylenediamine tetraacetic acid [Fe(III)-EDTA; Figure 1A], N-(2-hydroxyethyl) ethylenediaminetriacetic acid [Fe(III)-HEEDTA; Figure 1B], and di-ethylenetriamine pentaaetetic acid [Fe(III)-DTPA; Figure 1C], could alleviate Fe-deficiency, although this method is still not very common (Abadía et al., 2004). Trunk injection with liquid Fe fertilisers, or solid branch implants of Fe compounds are even less frequent, in spite of the long-lasting efficacy that can be obtained with one application per year (Abadía et al., 2004). The most widely-used Fe-fertilisation technique for fruit crops grown in calcareous soils is an annual soil application of expensive synthetic Fe(III)-chelates such as ethylenediamine-N,N'-di-(ortho-hydroxyphenyl) acetic acid [Fe(III)-o,o-EDDHA; Figure 1D] and analogues such as ethylenediamine-N-(ortho-hydroxyphenylacetic)-N'-

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(para-hydroxyphenylacetic) acid [Fe(III)-o,p-EDDHA; Figure 1E], ethylenediamine-N,N'-bis(2-hydroxy-4-methylphenylacetic) acid [Fe(III)-o,o-EDDHA; Figure 1F], ethylenediamine-N-(2-hydroxy-4-methylphenylacetic)-N'-(-4-hydroxy-2-methylphenylacetic) acid [Fe(III)-o,o-EDDHA; Figure 1G], ethylenediamine-N,N'-bis(5-carboxy-2-hydroxyphenylacetic) acid [Fe(III)-EDDCHA; Figure 1H], or ethylenediamine-N,N'-bis(2-hydroxy-5-sulphophenylacetic) acid [Fe(III)-EDDSHA; Figure 1I] (Lucena, 2006).

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

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

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

QUALITY OF SYNTHETIC Fe(III)-CHELATES

Commercial synthetic Fe(III)-chelate fertilisers are obtained by first synthesising the chelating agent, then incorporating Fe from an inorganic salt. The amount of synthetic Fe(III)-chelate in commercial formulations may be considered the main quality parameter. Several analytical techniques have been used to determine this value: paper, gel, or thin-layer chromatography, electrophoresis, gas chromatography, and high performance liquid chromatography (HPLC) have been used as separation techniques, combined to UV-VIS or atomic absorption spectroscopy (AAS) as detection techniques (see references in Álvarez-Fernández et al., 2007). Many of these methods were developed focussing on only one or a few synthetic chelates [mainly Fe(III)-EDTA]. Recently, more selective and sensitive analytical techniques such as inductively-coupled mass spectrometry (ICP/MS) and electrospray mass spectrometry (ESI/MS), coupled to HPLC, have been developed to determine, simultaneously, the levels of most commercial synthetic Fe(III)-chelates in environmental matrices (Álvarez-Fernández et al., 2007).

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

Some Official Analytical Methods have recently been approved to determine the parameters required by EU Regulations. The EU Standard EN 13366 (Anon, 2001a) describes the determination of total chelated Fe by AAS or ICP after separation of chelated Fe (anionic or neutral molecules) from non-chelated Fe (cations) in
a cation exchange column. This method has recently been compared unfavourably with an Association of Official Analytical Chemists (AOAC)-modified method based on the precipitation of inorganic forms at pH 9 (Vilén et al., 2007). Different official methods (EU Standards, EN) have been developed to determine Fe concentrations in soil solutions, and the extent of Fe(III)-chelate adsorption onto soil surfaces after Fe-fertilisation, were found between synthetic Fe(III)-chelates (even among isomers) depending on soil type. For example, the order of Fe(III)-chelate efficiencies were: EDDHSA > EDDHA > EDTA. The efficiency of a synthetic Fe(III)-chelate depends on its stability and persistence in the soil and in nutrient solutions, as well as on its ability to supply Fe to plants (Lucena, 2006). Stability and persistence may be assessed by competing metals, (ii) adsorption of the Fe(III)-chelate on soil surfaces, (iii) degradation of the Fe(III)-chelate or chelating agent, and (iv) lixiviation from the root-soil system. These processes have been studied by means of Fe speciation in soil solution using in silico calculations and also by direct determination of Fe(III)-chelates. In situ studies try to predict the amount of Fe bound to the chelating agent, based on soil solution pH, nutrient concentrations, and the protonation and stability constants of the chelates with Fe and other metals. The stability constants for most synthetic Fe(III)-chelates have been calculated using spectrophotometric data, after base titration with NaOH (Yunta et al., 2003), whereas the stability constants for many metal chelates have not yet been determined. In silico calculations can be put in question by the existence of kinetically slow metal-exchange reactions that would result in a non-equilibrium chemical speciation, and also by the presence of naturally occurring ligands that could compete with the chelating agents for available metals (Nowack, 2002). In situ data predict the substitution of Fe by Zn, Mn, or Ca in the case of Fe(III)-EDTA, but not in the case of Fe(III)-o,o-EDDHA at pH values above 6 (Lucena, 2006), which is in agreement with the high efficacy of Fe(III)-o,o-EDDHA in calcareous soils.

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Studies using direct determinations in soil solutions usually measure Fe(III)-chelates by UV/Vis, with or without a previous chromatographic separation, and soluble Fe by AAS and ICP. Differences in the concentrations of soluble Fe and Fe(III)-chelate in soil solutions, and the extent of Fe(III)-chelate adsorption onto soil surfaces after Fe-fertilisation, were found between synthetic Fe(III)-chelates (even among isomers) depending on soil type. For example, the order of Fe(III)-chelate efficiencies were: EDDHSA > EDDHA > EDTA. The ability of Fe(III)-chelates to supply Fe to Fe-deficient plants is probably the most studied aspect related to efficacy. Leaf chlorophyll content is the method best suited to assess plant Fe status (Abadía et al., 2004). However, other parameters such as plant biomass and Fe concentrations in leaves are also widely used to evaluate the efficiency of Fe fertilisers. However, these parameters do not provide an unequivocal assessment of the Fe status of the plant. In some cases, similar leaf Fe contents have been found in Fe-deficient and Fe-sufficient leaves. This is called the “chlorosis paradox” (Morales et al., 1998). Biomass and chlorophyll concentrations can also be affected by other biotic or abiotic stresses. The time-dependence of re-greening of Fe-chlorotic leaves is often used as a parameter to assess fertiliser efficiency. Determination of leaf re-greening is traditionally based on UV/Vis chlorophyll analysis of organic solvent extracts of leaves, although this is being substituted by other methods that use immediate, non-destructive, in vivo estimations (e.g., SPAD; a Soil Plant Analysis Development).
MECHANISMS OF ACTION OF SYNTHETIC Fe(III)-CHELATES

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

After the reduction of Fe(III) by FCR, the chelating agent remains in the soil solution, thus being able to dissolve native soil Fe and transport it to the plant rhizosphere. However, some studies have shown that whole Fe(III)-chelates, or synthetic chelating agents, can also be taken up by the plant. Fe(III)-EDDHA was found in Zinnia, sunflower, and soybean exudates (Tiffin et al., 1960), and in tobacco leaves (Jeffreys and Wallace, 1968) using UV/Visible. More recently, Fe(III)-o,o-EDDHA was found in tissues of tomato, pepper, and lettuce (Bienfait et al., 2004) using an excess of FeCl3 to ensure determination of the total amount of o,o-EDDHA as Fe(III)-o,o-EDDHA using HPLC-UV/Vis. Radioactivity assays with 57Fe(III)-o,o-EDDHA (Tiffin et al., 1961) and 59Fe(III)-o,o-EDDHA (Römheld and Marschner, 1981) confirmed plant uptake of EDDHA and Fe(III)-EDDHA in soybean, sunflower, pea, peanut, and some grasses (e.g., millet, wheat and corn). No differences in 14C uptake between Fe-deficient and Fe-sufficient plants were found. However, the 57Fe/59Fe ratios were 250-fold higher in Fe-deficient vs. Fe-sufficient soybean exudates and sunflower shoots; whereas, in grasses, the 59Fe/14C ratio was always approx. 1.0, irrespective of plant Fe status. Grasses secrete low molecular weight compounds, phytosideropores, that bind specifically to Fe(III), then take up the whole Fe(III)-phytosideropore complex using a specific transport protein. Until now, it is not clear whether EDDHA/Fe(III)-EDDHA uptake in plants is through a passive or an active pathway.

Recent analytical technologies can help us to understand the mechanism of action of Fe(III)-chelates. The use of ICP/MS equipped with a collision cell allows the quantification of very low amounts of stable Fe isotopes in plant tissues (Rodríguez-Castrillón et al., 2008). Using 54Fe-, 57Fe-, and 59Fe-chelates makes it possible to distinguish between the Fe supplied by the chelate and naturally occurring 56Fe. When cucumber plants were treated with 59Fe(III)-o,o-EDDHA for 1 h, they took up 5 µg 57Fe g⁻¹ FW, with 82%, 16%, and 2% being allocated to roots, leaves, and stems, respectively (Rodríguez-Castrillón et al., 2008). In the same study, better Fe translocation was observed for 57Fe(III)-o,o-EDDHA than for 59Fe(III)-o,p-EDDHA. Another technique recently applied to Fe-plant research is HPLC-ESI/MS using high-resolution detectors such as time-of-flight (TOF) devices. This technique makes the determination of Fe(III)-chelates inside the plant more reliable, since both the mass/charge (m/z) ratio and the isotopic signature are specific parameters for each metal compound. Also, this technique provides better limits of detection than HPLC-UV/Vis [e.g., 15-fold better for Fe(III)-o,o-EDDHA]. For instance, Fe(III)-o,o-EDDHA was determined specifically in tomato xylem sap using HPLC-ESI/MS(TOF) (Alvarez-Fernández et al., 2007) and 57Fe(III)-o,o-EDDHA as an internal standard (Figure 3). The two stereoisomer forms of Fe(III)-o,o-EDDHA (racemic and meso) were separated chromatographically, thus allowing determinations of the amounts of the two isomeric forms inside the plant. The o,o-EDDHA bound to two different stable Fe isotopes can also be distinguished using this technique [see insets in Figure 3 for the signals at m/z values of 412.0 and 413.0 for 57Fe(III)- and 59Fe(III)-o,o-EDDHA, respectively]. This technique could also permit speciation of the forms of chelating agent (EDDHA) and metal (stable metal isotopes) in plant tissues.

ENVIRONMENTAL IMPLICATIONS OF SYNTHETIC Fe(III)-CHELATES

The environmental implications of the use of synthetic Fe(III)-chelates have been less studied, and few studies are available on their environmental persistence, degradation, and toxicity. Photodegradation studies on Fe(III)-chelates were carried out for EDTA and DTPA,
soil layers with an excess of water (Lucena, 2003). Controls the movement of Fe(III)-chelates to the lower of Fe in leaves usually increases in plants treated with biomass, as well as changes in leaf mineral composition necrotic spots, leaf malformations, etc.) and a decrease in the order: Fe(III)-EDDHSA > Fe(III)-EDDCHA > Fe-DTPA, and slightly more toxic than FeSO₄.

The toxicological effects of EDDHA have been studied mainly in medical applications, when this chelating agent is used as a Fe chelating drug for patients with hemochromatosis. The median lethal dose of α,α-EDDHA (LD₅₀) was 53 mg kg⁻¹ for intervienal-treated rats and mice (Rosenkrantz et al., 1986), and 0.30 mg cm⁻² of soil for slugs¹ (Deroceras reticulatum) eggs exposed for 12 d (Iglesias et al., 2002).

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REFERENCES


FUTURE RESEARCH

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

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FIG. 3.

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

whereas only one recent study deals with the photochemical and redox behaviour of Fe(III)-α,α-EDDHA at different pHs (Gómez-Gallego et al., 2005). At typical environmental pH values (4 – 8), the low reduction potential of Fe(III)-α,α-EDDHA makes it unreactive in photochemically- or chemically-induced electron transfer processes, which invalidates photodegradation as an alternative mechanism for environmental elimination. The persistence of chelates in the soil depends on their polarity and solubility (following the order: Fe(III)-EDDHA > Fe(III)-EDDCHA >> Fe(III)-α,α-EDDHA > Fe(III)-α,α-EDDHEMA), and this controls the movement of Fe(III)-chelates to the lower soil layers with an excess of water (Lucena, 2003).

Phytotoxicity studies are less common, and have been based on the appearance of symptoms (e.g., necrosis, necrotic spots, leaf malformations, etc.) and a decrease in biomass, as well as changes in leaf mineral composition (e.g., Fe, Mn, Zn, Cu, P, etc.). Although the concentration of Fe in leaves usually increases in plants treated with Fe(III)-chelates, a poor correlation is commonly found between leaf Fe concentration and the severity of plant toxicity symptoms (Broschat and Moore, 2004). Fe(III)-EDDHA toxicity frequently causes a reddish stain in the foliage. In bean plants, the phytotoxic level in the nutrient solution was 4 mM Fe(III)-EDDHA (Wallace and Wallace, 1983); whereas African marigold and zonal geranium plants showed mild toxic effects at 1 mM with moderate toxic effects at 2 mM and 4 mM Fe(III)-EDDHA (Broschat and Moore, 2004). This study also found that Fe-EDDHA was less toxic than Fe-EDTA or Fe-DTPA, and slightly more toxic than FeSO₄.

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